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Novel diploids following chromosome elimination and somatic recombination in *Lolium multiflorum* x *Festuca arundinacea* hybrids

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Crosses were made between diploid *Lolium multiflorum* ($2n = 2x = 14$) and hexaploid *Festuca arundinacea* ($2n = 6x = 42$) in order to produce new forage hybrids. The F_1 were treated with colchicine to double the chromosome number and to restore fertility in the amphidiploids ($2n = 8x = 56$). Chromosome counting in fertile hybrids revealed some unexpected diploid plants ($2n = 14$), as well as the expected amphiploids with $2n = 56$. The phenotype of the diploids showed *Festuca*-like inflorescences which indicated they were not selfs of the *L. multiflorum* parent. Chromosome painting with a *L. multiflorum* genomic DNA probe showed the diploid complements to be recombinant and to carry some whole chromosomes, and some chromosome segments of *L. multiflorum* together with other chromosome parts which originated from the *F. arundinacea* genome. Meiotic behaviour of the diploids was regular, with seven bivalents. The results are discussed in terms of the segregation and recombination of different genomic components in the F_1 hybrids during diploidization.

Keywords: chromosome elimination, *Festuca arundinacea*, forage grasses, genomic *in situ* hybridization (GISH), *Lolium multiflorum*, somatic recombination.

Introduction

The *Lolium/Festuca* complex of forage grasses is cytogenetically amenable and has a broad range of agronomically useful and complementary genetic variation (Thomas & Humphreys, 1991). *Festuca arundinacea*, for example, has qualities of persistence during severe cold and drought and it complements the high nutritional value of *Lolium* species.

One way to exploit this useful resource is through interspecific hybridization followed by chromosome doubling to stabilize meiosis and to maintain fertility and the interspecific hybridity that comes from having disomic inheritance in amphiploids. This approach has resulted in a number of agricultural varieties developed from the *Lolium/Festuca* complex (Breese & Lewis, 1984; Zwierzykowski *et al.*, 1994). A second approach to manipulating variation is to exploit homoeologous recombination and to introgress desirable genes by hybridization and recurrent backcrossing, as demonstrated for the transfer of chromosome segments of *F. arundinacea* and *F. pratensis* into *L. multiflorum* (Humphreys & Thorogood, 1993; Humphreys & Ghesquière, 1994). The similar transfer has added drought resistance from *F. arundinacea* into *L. multiflorum* (Humphreys & Thomas, 1993; Humphreys & Pašakinskienė, 1996).

A breeding programme based on hybridization was started in 1989 at the Lithuanian Institute of Agriculture with the aim of combining the cold and drought resistance of *F. arundinacea* (*Fa*) with desirable forage traits of *L. multiflorum* (*Lm*). The F_1 hybrid *L. multiflorum* × *F. arundinacea* ($2n = 28$) is sterile, and fertility has to be restored by chromosome doubling to form the F_1C_0 amphidiploid, which

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is expected to give octoploids with $2n = 56$ chromosomes.

A population of F_1C_0 plants was produced and tested for anther dehiscence to evaluate their fertility. The surprising discovery was made that some of the fertile plants were not octoploid, as was expected, but were diploid with $2n = 14$. Observations on the phenotype of these diploids showed that they did not resemble either of the parents, but rather they displayed *Festuca*-like characteristics. It was also established that the diploids had a regular meiosis, with seven bivalents. The question therefore arises as to what is their origin? A fluorescent method of genomic *in situ* hybridization (GISH) can readily be used to distinguish the genomes of *Lm* and *Fa*, and the *Fa* itself has been shown to be a hybrid species deriving from *F. pratensis* (*Fp*, 2x) and *F. glaucescens* (*Fg*, 4x) (Humphreys *et al.*, 1995). The technique of GISH was therefore used to resolve this puzzle. The results reported here suggest that the diploids are recombinant and that they contain some *Lm* chromosomes, or *Lm* chromosome segments, together with other chromosome material from the *Fa* parental genome.

Materials and methods

Emasculated spikes of diploid *Lm* ($2n = 2x = 14$), var. 'Prima Roskilde' and var. 'S.22', were bagged and pollinated with the pollen of hexaploid *Fa* var. 'Barundi'-genotype no. 870, in the Spring of 1989. The hybrids required embryo rescue and were isolated after 16–18 days. Control F_1 embryos were cultured on Lismaier-Skoog medium (2/3 of salts) containing 30 g sucrose, 0.4 mg IAA, 0.25 mg kinetin and 7 g agar per litre. Embryos for colchicine treatment were grown on a medium with increased sucrose concentration at 70 g L^{-1}, and 3–8 mm seedlings were then treated with 0.3 per cent colchicine for a 4-h period. After treatment the F_1C_0 seedlings were maintained for 8–10 weeks on the same medium as the controls, and then transferred to soil in the glasshouse. In 1990 well grown plants were split into two clonal parts and transplanted to the field, and in 1991 the control F_1 and the F_1C_0 hybrids were tested for anther dehiscence to determine male fertility. Five clonal parts of each genotype of the fertile plants were then planted in an isolation plot to allow for cross-pollination and the production of the F_2C_1 generation. All fertile F_1C_0 hybrids were checked for chromosome number in root meristems, and later for their behaviour at meiosis, using standard cytological procedures.

Mitotic chromosomes from root tips were prepared for *in situ* hybridization by pretreatment in ice-water for 24 h before fixation, softened in a mixture of 2 per cent pectinase and 20 per cent cellulase and then squashed in 45 per cent acetic acid. Meiotic material was treated similarly, using meiocytes at the MI stage, but without pretreatment.

The protocol for genomic *in situ* hybridization (GISH) was described in Anamthawat-Jónsson & Heslop-Harrison (1996). To make the probe, total genomic DNA of *Lm* was sonicated to give fragments of 5–10 kb and then labelled with rhodamine-4-dUTP (Amersham) using the standard protocol for the nick translation system (Gibco BRL). Blocking DNA from *Fa* (200–500 bp fragments) was prepared by autoclaving for 5 min. The probe hybridization mixture (40 μL per slide) contained 100–200 ng labelled DNA, 4–8 μg blocking DNA, 50 per cent formamide in 2 × SSC (0.3 M sodium chloride, 30 mM trisodium citrate), 10 per cent dextran sulphate and 0.2 per cent SDS (lauryl sulphate). The hybridization mixtures were denatured by boiling for 5 min, then placed on ice. The chromosome substrates on the slides were denatured using 70 per cent formamide in 2 × SSC at 70°C for 2 min, dehydrated in ice-cold ethanol series (70, 90, 100 per cent, 2 min each) and then allowed to dry. Hybridization mixtures were applied to slides and incubated at 37°C overnight. Slides were washed with 20 per cent formamide in 0.1 × SSC at 42°C for 10 min and then rinsed three times in 2 × SSC. The slides were counterstained with DAPI and mounted in antifade (Vectashield, Vector Laboratories). The chromosomes were examined with the epifluorescence microscope.

Results

Breeding and phenotypes

In 1989 hybrids were produced between *Lm* ($2n = 14$) × *Fa* ($2n = 42$), and following embryo rescue the F_1 seedlings were grown on and treated with colchicine.

In 1991 control F_1 and F_1C_0 hybrid plants were tested for anther dehiscence, and the chromosome numbers of the fertile ones were counted (Table 1). Restoration of fertility was found for some of the F_1C_0 in both of the cross combinations tested, whereas all of the control F_1 were sterile with nondehiscent anthers (Table 1). The fertile F_1C_0 plants were mostly amphiploids with $2n = 56$ (24/30), but a smaller number unexpectedly turned out to be diploids with $2n = 14$ (6/30). These diploids were not selfs of *Lm*: on the contrary, all six had festucoid

Table 1 Numbers and percentages of plants with amphiploid and diploid chromosome numbers among the F_1 and F_1C_0 hybrids of *Lolium multiflorum* ($2n = 14$) × *Festuca arundinacea* ($2n = 42$)

			Hybrids with dehiscent anthers			
			Amphiploids $2n = 56$		Diploids $2n = 14$	
L. multiflorum × *F. arundinacea*		No. tested	No.	%	No.	%
var. Prima Roskilda × Barundi (genotype 870)	F_1	146	0	0	0	0
	F_1C_0	126	17	13.5	4	3.2
var. S22 × Barundi (genotype 870)	F_1	19	0	0	0	0
	F_1C_0	41	7	17.1	2	4.9

Table 2 Assessment for inflorescence phenotype in the F_2C_1 from the F_1C_0 diploids of *Lolium multiflorum* ($2n = 14$) × *Festuca arundinacea* ($2n = 42$)

			Plants with *Festuca* inflorescence		Plants with *Lolium* inflorescence	
Lm × *Fa*	Diploid F_1C_0 genotype	No. F_2C_1 plants	No.	%	No.	%
PR × 870	1804	51	48	94.1	3	5.9
	1637	68	68	100.0	0	0.0
	1787	52	41	78.8	11	21.2
S22 × 870	1731	75	75	100.0	0	0.0
	1771	78	77	98.7	1	1.3

inflorescences (panicles), and thus displayed the main and unambiguous distinguishing feature which clearly separates ryegrass (spike inflorescence) from fescue. However, some of these diploids, for example 1731, clearly showed semiperenniality and corresponded more with the *Lm* habit (*Festuca* is strongly perennial).

The F_2C_1 was produced in 1992 and was assessed for inflorescence phenotype in 1994. The results are given in Table 2. The *Lolium* inflorescence type is an indication of the hybrid origin of the F_1C_0 diploids, and was the most frequent in the F_2 descendants of F_1C_0 diploid 1787. Out of 52 plants studied, 11 were *Lolium*-like (21.2 per cent), whereas the other 41 were festucoid. Other F_1C_0 diploids gave a small number of *Lolium*-like descendants or none (Table 2). This result suggested that the F_1C_0 diploid genomes, although not being selfs, must nonetheless have contained variable amounts of genetic material from *Lm*.

GISH

Genomic *in situ* hybridization was used to test for the presence of *Lolium* DNA in the F_1C_0 diploid genotypes 1804, 1637, 1787 and 1731. Genotype 1787 is the most informative, and the GISH is shown in detail in Fig. 1. *Lm* total genomic DNA was labelled with rhodamine and hybridized with both mitotic and meiotic chromosomes. The control slide of mitotic cells was from the initial tetraploid F_1 hybrid, *Lm* ($2n = 14$) × *Fa* ($2n = 42$) (Fig. 1a,b). In this control the *Lm* probe hybridized essentially to the haploid set of seven *Lolium* chromosomes. A slight hybridization signal can be seen on seven *Festuca* chromosomes, presumably those of *Fp* origin, whereas no fluorescence is detected on 14 chromosomes of *Fg* origin. It is known that *Fp* is more closely related to *Lm* than it is to *Fg* (Humphreys & Ghesquière, 1994; Humphreys, 1995).

GISH of plant 1787 reveals mitotic cells with whole chromosomes as well as chromosome segments of *Lm* DNA (Fig. 1c,d), and confirms that the plant which gave the highest proportion of F_2 progeny with *Lm* inflorescences has a large amount of *Lm* DNA within its genome. The meiotic behaviour of all the diploids studied was normal, with seven bivalents, and the GISH of meiosis is shown in Fig. 1(e,f). The amount of *Lm* material was found to

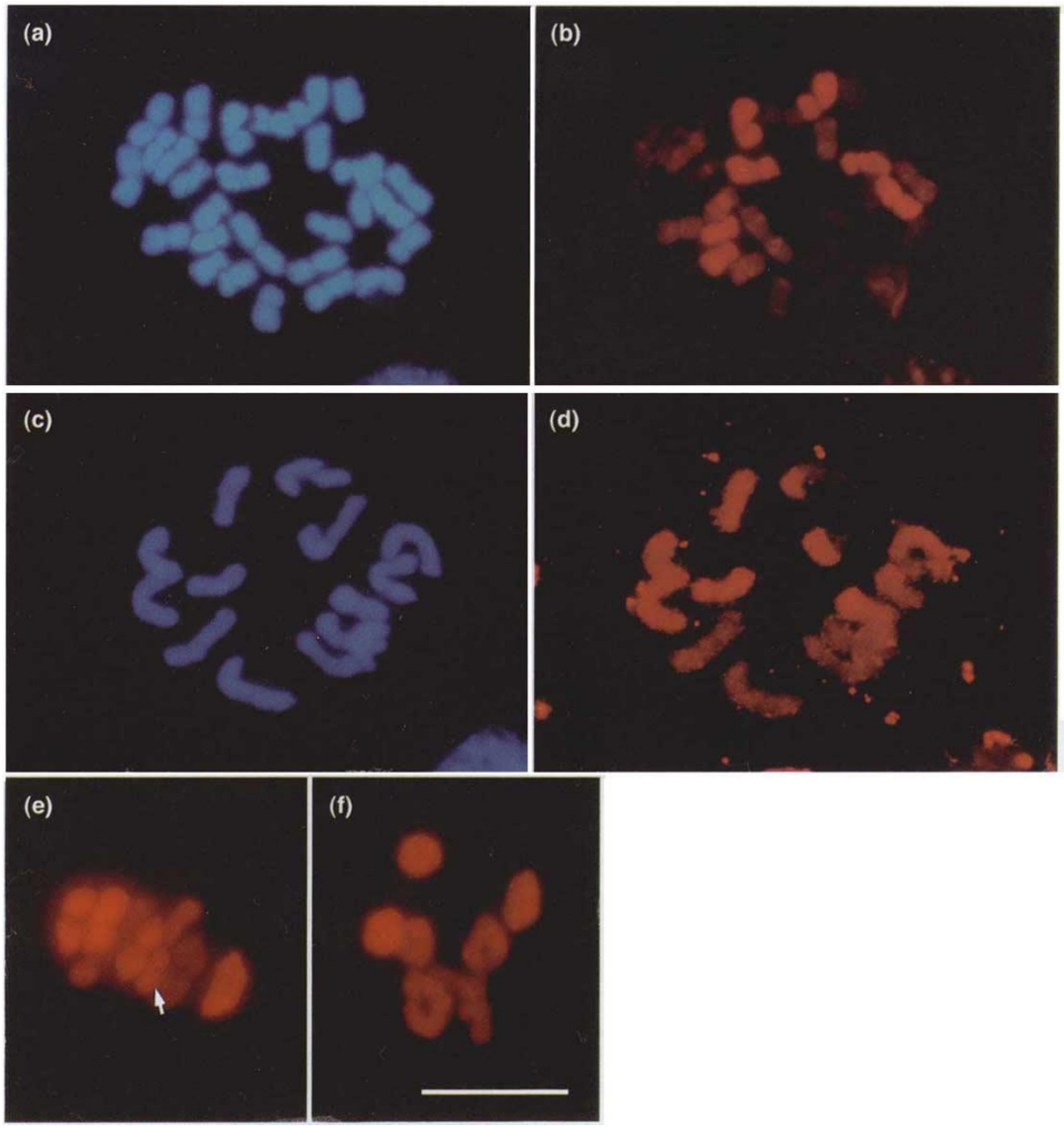

Fig. 1 GISH of rhodamine-labelled *Lolium multiflorum* (*Lm*) probe to chromosomes of *Lm* × *Festuca arundinacea* (*Fa*) hybrids. (a) DAPI-stained control of *Lm* × *Fa* F_1 hybrid ($2n = 28$) and, (b) the same cell showing seven rhodamine-labelled *Lm* chromosomes. (c) DAPI-stained *Lm* × *Fa* F_1C_0 diploid (1787) and (d) the same cell showing hybridization of *Lm* probe to whole chromosomes and some chromosome segments. (e) F_1C_0 diploid (1787) at metaphase I of meiosis showing bright fluorescence of *Lm* probe to four bivalents, and to a *Lm* chromosome in a heteromorphic bivalent (arrowed). (f) Another meiotic metaphase of the diploid 1787 showing two brightly fluorescing *Lm* bivalents. (Scale bar = 10 μm).

vary from cell to cell within this plant, in both the somatic and meiotic cells. One of the meiotic cells shown here (e) has four brightly labelled *Lm* bivalents and the other one (f) has two *Lm* bivalents. The complements are clearly recombinant with both *Lm* and *Fa* chromosomes, although, with the exception of the one heteromorphic bivalent (arrowed, (e)), there is a preference for individual bivalents to have both homologues as either *Lm* or *Fa*.

Discussion

The essence of the work reported here is that the amphidiploid from crossing diploid *Lm* ($2n = 14$) × hexaploid *Fa* ($2n = 42$) is unstable, and that in a proportion of the octoploid hybrids there is chromosome elimination and somatic recombination involving some whole and some parts of the constituent genomes. The outcome of these genomic inter-

actions is a number of diploids which carry some chromosomes or chromosome segments of *Lm* together with other chromosome material from the *Fa* genome. It is clear that the instabilities in the amphiploids have occurred after chromosome doubling, because the existence of *Lm* bivalents has been demonstrated. The possibility can be considered that the colchicine treatment itself, or the combination of colchicine, growth hormones and high sucrose concentration, may have triggered the instability in the amphiploids.

The diploids originate from only a small fraction of the amphiploids, 4/21 and 2/9, respectively, from the two cross combinations used (Table 1). Significant stability exists in these diploids in the sense that they generally have diploid chromosome numbers in all of their somatic cells and the chromosomes form seven bivalents in meiosis, albeit as 'new' and recombinant genomes. However, some karyotypic variation has been detected within these 'stable' diploid plants. This includes aneuploid ($2n = 12$) and haploid cells and some chromosome breakages, but these irregularities have not affected the fertility. We presume that any widely unbalanced intermediate chromosome numbers did not produce fertile plants and were therefore not detected.

It is not known when, after the doubling, the chromosome elimination has occurred in our hybrids. Genome elimination which takes place in wide species crosses, and which is routinely used for the production of dihaploids, occurs during early development within the embryo. This is so for example in *Hordeum vulgare* × *H. bulbosum* (Bennett *et al.*, 1976). What is different in the present work, however, is that elimination does not just involve whole genomes but compensating homologous chromosome partners as well.

The recombination process presumably occurs during elimination, and as we were only able to study mature plants there is no information on whether it happened stepwise over several nuclear divisions or not. In some cases (notably 1787) the diploids are a mosaic with different amounts of *Lm* chromosome parts in different cells, suggesting that 'balance' can arise in different ways, whereas in others the recombinants have stabilized in a constant form. The phenomenon includes genomic segregation as well as what appears to be somatic intrachromosomal recombination. Although the F_2C_1 have been assessed for phenotype, they have not been examined by GISH, and it is not known at this stage whether or not the passage through the sexual cycle will determine new and fixed genomic forms.

As far as ongoing developmental instability is concerned there is an interesting case for barley × rye hybrids, reported by Linde-Laursen in 1991. In this case the rye parents carried additional B chromosomes, and in the hybrids both the rye A and B chromosomes persisted into the seedling stage in some plants: the Bs were then lost completely by the end of 3 months, but the As survived in variable numbers up to the time of meiosis. This hybrid not only shows a unique aspect of differential genomic interaction between barley and between the A and B chromosomes of rye, but it also indicates that such interactions can persist and give rise to chromosome mosaics within plants.

In the situation of barley × rye, as well as many others in the past, it is not known whether somatic recombination may have been going on as well as elimination. An early indication that such somatic segregation may occur came from work of Sliesaravichus *et al.* (1986). These authors found a diploid genotype among the expected F_1 allotetraploids from the cross of *F. arundinacea* ($2n = 42$) × *F. pratensis* ($2n = 14$), but it was not possible to follow the work further at that time. Wilkinson *et al.* (1995), however, have recently described evidence for somatic translocation during potato dihaploid induction. Dihaploids of *Solanum tuberosum* are made by pollinating *S. tuberosum* with the 'dihaploid inducer' *S. phureja*, and, as Wilkinson and coworkers point out, euploid dihaploids have always been considered to be genetically pure and to be gametic samples of their tetraploid parents. A molecular study of the euploid dihaploid PDH55 revealed the expression of DNA from the inducer, and GISH analysis showed the inducer DNA to be present in the S. *tuberosum* chromosomes. The results were interpreted in terms of somatic translocation events occurring early on before the dihaploids were formed, and the patterns appear to be mitotically stable. The paper by Wilkinson and colleagues also contains a useful review and bibliography of somatic translocation events in other hybrids, most of which relate to plants grown from tissue culture.

Nucleo–cytoplasmic interactions, as defined by Gill (1991), offer one possible explanation for the phenomena described here. In the *Lm* × *Fa* amphiploid there are four different nuclear genomes involved (*LmLm FpFp* $FgFgFg_IFg_I$), three of which come from the *Fa* parent (*FpFp* $FgFgFg_IFg_I$), and which have presumably passed through the evolutionary 'bottleneck' crisis which affects new polyploids, and one of which is newly introduced (*LmLm*). In addition there is the cytoplasmic interaction between the *Fa* nuclear genomes and the *Lm* cytoplasm, so we have a juxtaposition of new and

untested combinations of nucleo–cytoplasmic conditions. It may not be so surprising therefore that some of these new amphiploids are unstable, and that they resolve their instability in this rather dramatic way. This speculation still leaves unresolved, however, the question of how most of the amphiploids survive whereas others diploidize themselves, and there is no answer to this point at present.

The work is now concentrated, amongst other things, on differentiating the *Fa* genomes present in these diploids. Preliminary results suggest the *Fp* genome to be preferentially retained and *Fg* eliminated.

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