Theor Appl Genet (1988) 76:299–304

The use of a triploid hybrid for introgression in *Lolium* species

H. Thomas, W. G. Morgan and M. W. Humphreys

AFRC Institute for Grassland and Animal Production, Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, Dyfed, UK

Received February 8, 1988; Accepted March 8, 1988
Communicated by J. Mac Key

Summary. Triploid hybrids of *Lolium multiflorum* (4x) × *L. perenne* (2x) behaved cytologically as autotriploids but the segregation of isozyme variants did not always agree with the expected trisomic ratios. The overall effect of these deviations from expectation was a greater proportion than expected of diploid progeny from the cross *L. multiflorum* (2x) × triploid hybrid which did not include any of the *L. perenne* alleles at the three marker isozyme loci. The possible mechanisms for these aberrant segregation ratios are discussed together with the advantages of the crossing scheme to accelerate the recovery of the genotype of the recurrent parent in a backcrossing programme to transfer characters from one species to another.

Key words: *Lolium* – interspecific hybrids – backcrossing – genetic segregation – introgression

Introduction

The scope for the use of interspecific hybrids in the breeding of improved forage grasses has been realised for a number of years (Breese and Lewis 1984) and the main effort has concentrated on breeding fertile and stable amphiploids which include the complete genomes of two species. Such breeding programmes attempt to combine the complementary attributes of two parental species in a synthetic amphiploid as in the tetraploid hybrids combining the genomes of the closely related diploid species *Lolium multiflorum* and *L. perenne* (Breese et al. 1981). The commercial exploitation of this particular amphiploid was made possible because it was sufficiently stable to retain most of the hybrid characteristics for the generations required to multiply the seed stocks for the release of the cultivars. This was primarily due to the meiotic behaviour of the amphiploids where a degree of preferential chromosome pairing leading to a mode of inheritance varying from disomic to polysomic slows down the segregation of species characters and hence conserves advantageous hybrid gene combinations (Breese et al. 1981).

An alternative approach to amphiploid breeding would be to transfer characters from one species to another, which can be achieved by a backcrossing programme. In the perennial forage grasses where only one generation per year is possible any backcrossing programme would be a protracted process. Using isozyme variants as genetic markers, Tanksley et al. (1981) demonstrated that it should be possible to reduce the number of backcrosses required to re-establish the recipient genotype including the transferred gene. They exploited natural differences in isozymes between the donor and recurrent parent in crosses between *Lycopersicon esculentum* and *Solanum penelli* which were used as markers of the genomes of the two species. The backcross (BC) progeny which were closer to the recurrent species in terms of a range of isozymes were also closer to the mean values of the recurrent parents for a number of quantitative characters. The ability to identify genotypes resembling the recurrent parent as young seedlings would be an advantage in a BC programme because it would allow the breeder to concentrate on fewer plants for succeeding backcrosses and possibly reduce the number of backcrosses required to restore the genotype of the recurrent parent.

An extension of this scheme using isozyme variants would be to impose some restriction on the recombination of species characters which would facilitate a speedier recovery of the genotype of the recurrent parent. Establishing a situation where a certain degree of preferential chromosome pairing is possible could be a method of restricting recombination. In the present experiment

triploid hybrids, in which the diploid complement of *L. multiflorum* chromosomes is combined with the haploid set of *L. perenne*, were made as the initial hybrid in a study to investigate the introgression of characters of *L. perenne* into *L. multiflorum*. Both genomes were marked by isozyme variants on three different chromosomes and this paper describes an analysis of the progenies of the first backcross generation.

Materials and methods

The triploid hybrids were produced by crossing a tetraploid line of *L. multiflorum* with a diploid genotype of *L. perenne*. The embryos were excised 15–18 days after pollination and cultured on a modified Gamborg and Miller's B5 medium (without 2,4 D and kinetin but including 3% sucrose). The genotypes of the 2x and 4x parents with regard to three enzyme systems, phosphogluco-isomerase (PGI/2), glutamate oxaloacetate transaminase (GOT/3) and superoxide dismutase (SOD/1) were selected to ensure that the *L. perenne* (*Lp*) allele could be distinguished in all progeny arising from crosses involving the triploid hybrid. Two appropriate triploids P122/6/12 and P122/6/16 were identified from the following 4x × 2x crosses:

	PGI/2	GOT/3	SOD/1
L. perenne (2x)	bb	ab	bb
L. multiflorum (4x)	bddd	bbbb	aaaa
Triploid P122/6/16	ddb	bba	aab
Triploid P122/6/12			

For the PGI/2 locus it was possible to identify plants with the double dose of the 'd' allele because of the greater intensity of the band. The triploid hybrid has the *L. perenne* chromosomes marked by the 'b' allele at the PGI/2 locus, the 'a' allele at the GOT/3 locus and the 'b' allele at the SOD/1 locus.

The third triploid P122/5/2 derived from the following 4x × 2x cross:

	PGI/2	GOT/3	SOD/1
L. perenne (2x)	bb	ab	bb
L. multiflorum (4x)	aabd	bbbb	aaaa
Triploid P122/5/2	adb	bba	aab

The alleles at the GOT/3 and SOD/1 loci were the same as the other two triploids but differed at the PGI/2 locus (adb) with the *L. perenne* chromosome marked by the 'b' allele.

The triploid hybrids were sufficiently male fertile to be crossed reciprocally with diploid *L. multiflorum* lines. The *L. multiflorum* genotypes used in the backcrosses (BC) were selected so that the *Lp* alleles of the triploids could be readily identified in the BC progeny. The chromosome number of the BC progenies was determined and all the euploid (2n = 14) seedlings retained. The phenotype of all the euploid BC seedlings was scored for all three enzyme systems using gel electrophoresis techniques (Hayward and McAdam 1977; Humphreys 1984).

For mitotic analyses root tips were collected, pre-treated in chilled distilled water at 1°–2°C, fixed in a solution of 3 parts alcohol to 1 part acetic acid, hydrolysed in NHCl at 60°C for 10 min and stained in Feulgen solution. The root tips were squashed in 45% acetic acid and the chromosome number determined. For meiotic studies immature spikes were fixed in 6:3:1 solution of ethyl alcohol, chloroform and acetic acid. Anthers were stained in alcoholic hydrochloric acid-carmine (Snow 1963) and squashed in 45% acetic acid.

Results

Chromosome pairing in the triploid hybrids

Chromosome pairing in the triploids *Lm Lm Lp* was typical of an autotriploid with a high frequency of trivalents (Table 1). The maximum pairing of 7 trivalents was recorded in 3 out of the 64 PMCs scored in the two hybrids P122/5/2 and P122/6/12. The third triploid had 20 chromosomes only but it also had a mean of 5.13 trivalents. The mean number of open chain trivalents was 2.32 compared with 2.56 pan-handle configurations. There was little evidence of the preferential pairing of the homologous pairs of the *L. multiflorum* chromosomes since the frequency of cells with only bivalents and univalents was low.

Chromosome numbers of the progeny of the L. multiflorum × *triploid hybrid*

The triploid hybrids were sufficiently male fertile to result in the dehiscence of the anthers and they could be used as either the male or female parents in crosses with *L. multiflorum*. In the cross using the triploid as the female parent the majority of the progeny were aneuploids (Table 2). Theoretically it should be possible to recover plants with chromosome numbers ranging from 2n = 14–21 however, the only progeny with more than 18 chromosomes found was a single seedling with 23 chromosomes.

In the reciprocal cross using the triploid hybrid as the pollen plant 87.69% of the progenies were euploid plants with 14 chromosomes. The preponderance of euploid progeny indicates that pollen with the haploid number of 7 chromosomes had a selective advantage over aneuploid gametes, a feature commonly observed in aneuploid

Table 1. Chromosome pairing in triploid hybrids *L. multiflorum* (4x) × *L. perenne* (2x)

Plant no.	Mean number of III	II	I	Chiasmata
P122/6/12	5.00 (2–7)[a]	2.00 (0–5)	2.00 (0–5)	16.37
P122/5/2	4.53 (1–7)	2.47 (0–6)	2.47 (0–6)	15.70
P122/6/16	5.13 (3–6)	1.87 (1–4)	0.87 (1–3)	16.10

[a] Figures in brackets are the ranges

Table 2. The chromosome number of progeny from reciprocal crosses between the triploid hybrid and *L. multiflorum*

Cross	Chromosome number					
	14	15	16	17	18	21
3x × 2x	23 (29.11)[a]	34 (43.04)	18 (22.78)	2 (2.53)	1 (1.26)	–
2x × 3x	741 (87.69)	94 (11.12)	7 (0.80)	1 (0.12)	–	1 (0.12)

[a] Figures in brackets are percent

Table 3. Estimated frequencies of gametes with different chromosome number in the triploid hybrid

	Chromosome number					
	7	8	9	10	11	12
Expected	16 (8.33)[a]	62 (32.29)	52 (27.08)	44 (22.91)	16 (8.33)	2 (1.04)
Observed	23 (29.11)	34 (43.04)	18 (22.78)	2 (2.53)	1 (1.26)	–

[a] Figures in brackets are percent

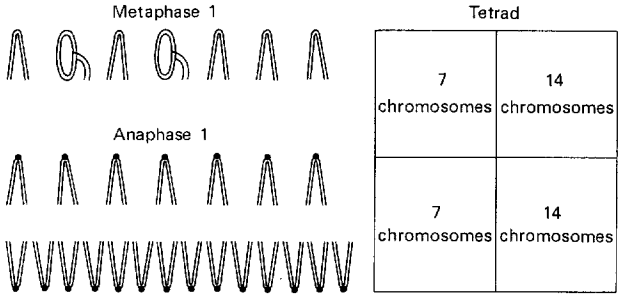

Fig. 1. Diagrammatic representation of metaphase 1 and anaphase 1 which could result in euhaploid gametes

plants (Khush 1973). Although the selective advantage of the euhaploid pollen is indisputable the chromosome pairing observed in the triploid does not readily indicate that a large proportion of the gametes formed are euhaploid. Cells in which $7_{II}+7_{I}$ are formed with the lagging univalents being lost during meiosis would be the most obvious source of euhaploid gametes but not a single PMC with the maximum possible number of univalents was recorded. However, it is possible that a PMC with 7 trivalents all orientated in the same direction on the metaphase plate (Fig. 1), would give rise to a 7–14 separation at anaphase 1.

The orientation of trivalents in PMCs at metaphase 1 were scored in 50 cells and assuming that the multivalents would remain at that orientation and separate accordingly at anaphase, and that univalents would be lost as micronuclei during meiosis, an estimate of the frequency of gametes with 7–14 chromosomes was made and presented in Table 3. Only 8.33% of the gametes formed were expected to be euhaploid.

In the cross using the triploid hybrid as the female parent there would be no comparable selective advantage to the euhaploid gametes as occurs in the reciprocal cross. The distribution of the gametes with different chromosome number can be obtained by substracting the 7 *L. multiflorum* chromosomes from the numbers of the 3x × 2x progeny. The frequencies of the different chromosome number classes differed significantly from the expectation based on the calculated frequencies (Table 3). The main discrepancy between the observed and calculated frequencies is the excess of euhaploid gametes and a deficiency of gametes with 9, 10 and 11 chromosomes. The significant difference between the observed and calculated expectations could reflect errors in the method used to calculate the chromosome number of the gametes or inviability of zygotes with the higher chromosome numbers.

Segregation of isozyme variants in progenies of L. multiflorum × *triploid hybrids*

If each of the chromosomes in the sets of three in the triploid hybrid are completely homologous all three alleles would have an equal chance of being included in the euhaploid gametes formed. Therefore as long as the position of the three chromosomes is at random in the trivalents there is a 1 in 3 chance of the gametes formed by the triploid containing the *Lp* allele and the expected ratio in the *L. multiflorum* × triploid progeny would be 2 *Lm*: 1 *Lp*.

All the euploid progeny from the *L. multiflorum* × triploid BC progenies were scored for their phenotype with regard to the three enzyme systems. In Table 4 the data are presented as the number of progeny observed with the *Lm* and *Lp* allele for the PGI/2, GOT/3 and SOD/1 loci.

All the three hybrids showed a significant deficiency of the *Lp* PGI/2 allele as did the pooled data for the three hybrids. The heterogeneity X^2 was not significant.

The pooled data for the GOT/3 did not deviate from the expected ratio but the heterogeneity X^2 was significant. The cross involving P122/6/12 was in agreement with the 2:1 ratio but the P122/6/16 cross yielded a significant excess of progeny with the *Lp* allele. In the P122/5/2 cross there was a significant deficiency of the *Lp* allele.

Again the main feature of the SOD/1 data was the significant heterogeneity between the three crosses, P122/5/2 showed a significant excess of the *Lp* allele in marked contrast to a 45% deficiency of the *Lp* allele in P122/6/12 and 32% in P122/6/16.

Table 4. Segregation in the diploid progeny from *L. multiflorum* × triploid hybrid crosses

Cross	Lp	Lm	χ^2
PGI/2			
P122/6/12	92	497	82.70
P122/6/16	27	305	94.55
P122/5/2	29	129	15.97
Total	148	931	183.35
Heterogeneity χ^2 $P = 0.20–0.10$			
GOT/3			
P122/6/12	174	392	1.72
P122/6/16	144	188	12.96
P122/5/2	21	137	28.55
Total	339	717	1.04
Heterogeneity χ^2 $P < 0.001$			
SOD/1			
P122/6/12	109	483	58.92
P122/6/16	75	257	17.54
P122/5/2	67	90	6.21
Total	251	830	49.48
Heterogeneity χ^2 $P < 0.001$			

The objective of the experiment was to assess the possibility of reducing recombination and accelerate the recovery of the genotype of the recurrent parent in a BC programme; therefore the number of progeny with the different combinations of the three loci were recorded. The realised frequencies of the *Lp* alleles at each of the three loci were used to calculate the expected frequencies of the eight possible genotypes including all combinations of alleles at the three loci. The observed and expected frequencies are presented in Table 5. The pooled data over the three crosses agreed with expectation indicating that the three loci behaved independently and the frequencies of the combinations of the three loci are as predicted on a random basis. However, the heterogeneity X^2 showed that there were significant differences between the three crosses with P122/6/12 and P122/6/16 showing significant deviations from the calculated expected ratios.

Discussion

Interspecific hybridisation is a means of extending the range of variation beyond that encompassed by the parental species. Provided the parental species are interfertile backcrossing is the most plausible method to transfer desirable variation from one species to another. According to Allard (1960) up to six backcrosses are usually required to recover the genotype of the recurrent parent and in a perennial forage crop with specific vernalisation and light requirements for floral initiation, where only one generation per year is possible, the procedure is protracted. Tanksley et al. (1980, 1981) have shown that by using isozymic variation between *Lycopersicon esculentum* and *Solanum pennelli* to mark the specific genomes it should be possible to reduce the number of backcrosses required. Seedlings which are homozygous for the major-

Table 5. Expected and observed progenies of the cross *L. multiflorum* × triploid hybrids in relation to their genotype for the three marker loci

Genotype			Cross			Totals
PGI	GOT	SOD	P122/5/2	P122/6/16	P122/6/12	
+[a]	–	–	14 (13.86)[b]	9 (29.03)	51 (20.23)	74 (76.82)
–	+	–	8 (12.13)	100 (69.97)	121 (178.60)	229 (225.14)
–	–	+	51 (46.07)	36 (35.62)	68 (68.03)	155 (146.18)
+	+	–	3 (2.72)	8 (12.94)	23 (15.55)	34 (35.21)
+	–	+	8 (10.32)	4 (6.59)	7 (5.90)	19 (23.47)
–	+	+	9 (9.04)	32 (15.79)	21 (53.18)	62 (68.29)
+	+	+	1 (2.02)	3 (2.68)	6 (4.51)	10 (10.67)
–	–	–	64 (61.84)	139 (157.99)	281 (232.93)	484 (482.28)
			$\chi^2 = 3.010$ $P = 0.90–0.80$	$\chi^2 = 48.163$ $P < 0.001$	$\chi^2 = 97.349$ $P < 0.001$	$\chi^2 = 2.224$

[a] + denotes presence of Lp allele
[b] Figures in brackets are the expected numbers

ity of isozymic alleles of the recurrent parent could be selected for backcrossing and would lead to a quicker recovery of the genotype of the recurrent species. The restriction on recombination imposed by the polyploid structure of the triploid hybrids expedites further the recovery of the genotype of the recurrent parent.

Using three marker loci in a diploid backcross the proportion of progeny expected without any of the *Lp* alleles would be 0.125 compared with 0.296 from the triploid backcross. These frequencies are based on each chromosome of the sets of three being completely homologous and behaving like primary trisomics. Since each locus can be assumed to mark a segment of chromosome, scoring for the absence of the *Lp* alleles is a measurement of the recovery of the recurrent *L. multiflorum* genotype. Therefore, even when the triploid hybrid behaves meiotically as an autotriploid a smaller BC population would be required to isolate genotypes similar to the recurrent parent at these particular loci than in a diploid backcross. When the realised data for each of the three loci were used to calculate the expected frequencies of the different combinations involving all three loci the proportion of the progeny expected without one of the *Lp* alleles is 0.452 (Table 5). This represents a 52% increase over the theoretical expectation from a cross involving an autotriploid.

Cytologically the triploid hybrids showed a typical autotriploid behaviour, but the segregation ratios obtained for the *Lp* PGI/2 and the *Lp* SOD/1 b alleles indicated degrees of preferential chromosome pairing because there was a deficiency of progeny including the *Lp* alleles. Since the loci mark different chromosomes of the genome the variation between loci indicate that differentiation leading to preferential pairing was not distributed evenly throughout the genome. Some chromosomes have a greater potential to pair preferentially than others. Although Breese and Thomas (1977) reported 33% preferential pairing when they used PGI/2 locus as the genetic marker Lewis (1981) found less evidence of preferential pairing when he used the 'non red' base character as a genetic marker in *L. perenne* × *L. multiflorum* tetraploid hybrids. The significant deviation at the PGI/2 locus confirms the data of Breese and Thomas (1977) that a degree of preferential pairing could be demonstrated using this particular locus. The heterogeneity between the crosses using the three triploid genotypes was significant for the GOT/3 and SOD/1 loci and for the overall frequencies involving the combinations of the three loci. Variation in the control of preferential chromosome pairing between plants within populations of the tetraploid hybrids *L. perenne* × *L. multiflorum* has been demonstrated by Lewis (1981) and this probably accounts for the heterogeneity of the segregation ratios in the BC progeny.

Euhaploid (n = 7) pollen had a selective advantage over gametes with more than the haploid number of chromosomes in the *L. multiflorum* × triploid hybrid crosses (Table 2). The frequency of the haploid gametes formed was not only dependant on the number of trivalents within a PMC but also the orientation of the trivalents within a cell. As a consequence of the failure of the *L. perenne* chromosome to form a chiasmate association with the homologous pair of *L. multiflorum* chromosomes the *L. perenne* univalent would have a high probability of being lost during the later stages of meiosis. This would be a contributory factor in the significant deficiency of the *L. perenne* allele within the progeny from *L. multiflorum* × triploid crosses. The position of the *L. perenne* chromosome within a trivalent and the orientation of the trivalent on the metaphase plate could also have a significant effect on the incorporation of the *Lp* allele in the haploid gamete. If the position of the *Lp* chromosome within the trivalent was at random all three chromosomes would have an equal chance of being included in the haploid gametes. However, if the *Lp* chromosome was one of the terminal chromosomes in an orientated chain trivalent or the handle in a pan-handle configuration, the chances of the chromosome being included in any balanced haploid gamete would be reduced (Fig. 1). Since the central chromosome of the chain trivalent or one of the chromosome forming the ring in a pan-handle configuration would be positively selected for in the haploid gametes the inclusion of the *Lp* allele would be dependant on recombination between the *Lp* and *Lm* loci. Only one of the arms of the *Lp* chromosome would be paired with the *Lm chromosome* and assuming that each arm had an equal chance of pairing this would reduce the probability of the inclusion of the *Lp* allele in a balanced haploid gamete by a half and could account for the deficiency of the *Lp* allele in the diploid × triploid progeny. However, if the chromosome arm carrying the *Lp* marker gene paired at a frequency greater than random the chances of recombination would be increased. The *L. multiflorum* chromosome would be at a selective advantage in the formation of euploid gametes and the recombined *Lp* allele would be positively selected and this could account for the significant excess of the *Lp* GOT/3 and SOD/1 alleles in some crosses. The variation between triploids probably reflects genotypic differences in the control of meiotic behaviour as reported by Lewis (1981).

The use of the triploid hybrid does provide an opportunity for controlled introgression of genes and gene complexes between the two species *L. perenne* and *L. multiflorum*. The two species have a number of complementary attributes that would be desirable to incorporate in stable hybrid derivatives and although the species are interfertile producing fertile F_1 and F_2, there is evidence of genetic deterioration in later generations with varying degrees of sterility (Naylor 1960). Using the isozyme variants as genetic markers of the two diploid genomes in

the triploid hybrid it has been shown that the recovery of the recurrent genotype could be accelerated and the number of backcrosses reduced. The procedure would provide the opportunity to select at the seedling stage progenies resembling the recurrent species and would reduce the number of plants to be assayed as established plants for introgressed characters. Only 3 of the 7 chromosomes were marked and the efficiency of the system in accelerating the recovery of the recurrent genotype would be increased if all the chromosomes had specific markers. Tests would also have to be undertaken to determine whether the gene or gene complex to be transferred were linked with any of the isozyme loci. Linkage could be advantageously used because selection for agronomic characters could be based on the seedling genotype at the isozyme locus.

Acknowledgements. We would like to thank Dr. E. L. Breese for valuable discussions and comments.

References

Allard RW (1960) Principles of plant breeding. Wiley, London New York

Breese EL, Thomas AC (1977) Monitoring chromosome segregation in *Lolium multiflorum* × *L. perenne* amphiploids. In: Rep Welsh Pl Breed Stn for 1976, pp 29–32

Breese EL, Lewis EJ (1984) Breeding versatile hybrid grasses. Span 27:3–5

Breese EL, Lewis EJ, Evans GM (1981) Interspecies hybrids and polyploidy. Philos Trans R Soc London Ser B 292:487–498

Hayward MD, McAdam NJ (1977) Isozyme polymorphism as a measure of distinctiveness and stability in cultivars of *Lolium perenne*. Z Pflanzenzücht 79:59–68

Humphreys MW (1984) Superoxide dismutase (SOD) in *Lolium/Festuca* species. In: Rep Welsh Pl Breed Stn for 1984, pp 100–101

Khush GS (1973) Cytogenetics of aneuploids. Academic Press, New York London

Lewis EJ (1981) Genetic segregation in relation to chromosome pairing in tetraploid hybrids between *Lolium perenne* and *L. multiflorum*. Theor Appl Genet 59:139–143

Naylor B (1960) Species differentiation in the genus *Lolium*. Heredity 15:219–233

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosome squash preparations. Stain Technol 38: 9–13

Tanksley SD, Rick CM (1980) Isozymic gene linkage map of the tomato: applications in genetics and breeding. Theor Appl Genet 57:161–170

Tanksley SD, Medina-Filho H, Rick CM (1981) The effect of isozyme selection on metric characters in an interspecific backcross of tomato – basis of an early screening procedure. Theor Appl Genet 60:291–296