Chromosoma (1990) 99:289–295

CHROMOSOMA

Diploidisation of *Lotus corniculatus* L. (Fabaceae) by elimination of multivalents

A. Davies, G. Jenkins, and H. Rees

Department of Agricultural Sciences, U.C.W. Aberystwyth, Penglais, Aberystwyth, Dyfed, SY23 3DD, UK

Received February 12, 1990 / in revised form March 23, 1990
Accepted April 11, 1990 by P. Moens

Abstract. *Lotus corniculatus* L. (Fabaceae) is a natural tetraploid of probably hybrid origin, which regularly forms bivalents at metaphase I of meiosis. Whole-mount surface-spreading of synaptonemal complexes (SCs) under the electron microscope reveals that diploidisation of this species is achieved not by exclusive pairing of homologues during meiotic prophase, but by the elimination of multivalents in favour of bivalents before metaphase I. Observations show that 43% of multivalents are eliminated between zygotene and pachytene, presumably by dissolution and reassembly of SCs between homologous chromosomes. A further 63% are eliminated between pachytene and diakinesis, with a commensurate increase in the number of univalents. Elimination ensures few multivalents reach first metaphase and effectively diploidises this tetraploid.

Introduction

Rasmussen and Holm (1979) reported that in achiasmate autotetraploid females of *Bombyx mori* exclusive bivalent formation at first metaphase was achieved not by strict two-by-two pairing during zygotene, but rather by elimination of multivalents during pachytene. Rasmussen (1987) later showed in chiasmate autotetraploid males that the elimination process was effectively impeded by crossing over and the formation of chiasmata, which led inevitably to the persistance of multivalents to metaphase I. Since its discovery in silkworms, the elimination process has been shown to be a means of bivalent formation in other polyploid species, which have to contend with the inhibiting effects of chiasma formation (for list see Jenkins and White 1990). For example, detailed and exhaustive studies of chromosome pairing behaviour in allohexaploid wheat, *Triticum aestivum*, have revealed that multivalent formation is a regular feature of zygotene (Hobolth 1981; Jenkins 1983; Holm 1986). By pachytene, however, the vast majority of multivalents are transformed into homologous bivalents before crossing over, a process ensuring the regular segregation of chromosomes and disomic inheritance of the organism (Holm and Wang 1988). Similarly, both natural and artificial polyploids of *Scilla autumnalis* form multivalents during zygotene, but largely eliminate them before crossing over occurs (White et al. 1988; Jenkins et al.1988).

Lotus corniculatus is a natural tetraploid of the Fabaceae, which forms bivalents at metaphase I of meiosis. It was selected for study primarily to determine whether or not this dicotyledonous species eliminates multivalents, as in the several monocotyledons examined to date. Furthermore, it provides the technical advantage of having a small number ($2n=4x=24$) of small chromosomes, which permits a sufficiently large number of cells to be studied in order to monitor precisely changes in synaptic behaviour throughout meiotic prophase. It

Table 1. Total lateral component (*LC*) length (including quadrivalents), percentage pairing of bivalents and number of bivalents (*II*) and quadrivalents (*IV*) in the 13 zygotene nuclei

Nucleus	LC length (μm)	Percentage pairing	II	IV
1	581	47	12	0
2	428	60	10	1
3	451	60	10	1
4	555	83	10	1
5	486	85	10	1
6	490	87	10	1
7	532	92	6	3
8	499	93	10	1
9	438	95	10	1
10	631	98	8	2
11	367	98	12	0
12	412	98	10	1
13	488	98	6	3
Total			124	16
Mean/nucleus			9.53	1.23

Offprint requests to: G. Jenkins

was hoped, also, that observations of meiotic chromosome interactions would reflect the structural and genetic relatedness of the two constituent genomes, and provide new insights into its controversial phylogenetic origin, polyploid status and mode of inheritance. On the basis of genetical and cytological observations, Dawson (1941) proposed that *L. corniculatus* is an autotetraploid of *L. tenuis* showing tetrasomic inheritance. Larsen (1954) forwarded *L. alpinus* on morphological grounds as the more likely diploid ancestor, and Wernsman et al. (1964) used cytological evidence alone apparently to confirm the autopolyploid status of *L. corniculatus*. However, since then detailed observations of the segregation of isoenzyme alleles has shed doubt upon the validity of these conclusions (Raelson and Grant 1988; Raelson et al. 1989). Evidence of quadruplication of loci in *L. corniculatus* has been taken to mean that the original duplication in the diploid ancestor resulted from unequal crossing over between homoeologues. This suggests that *L. corniculatus* is a segmental allotetraploid,

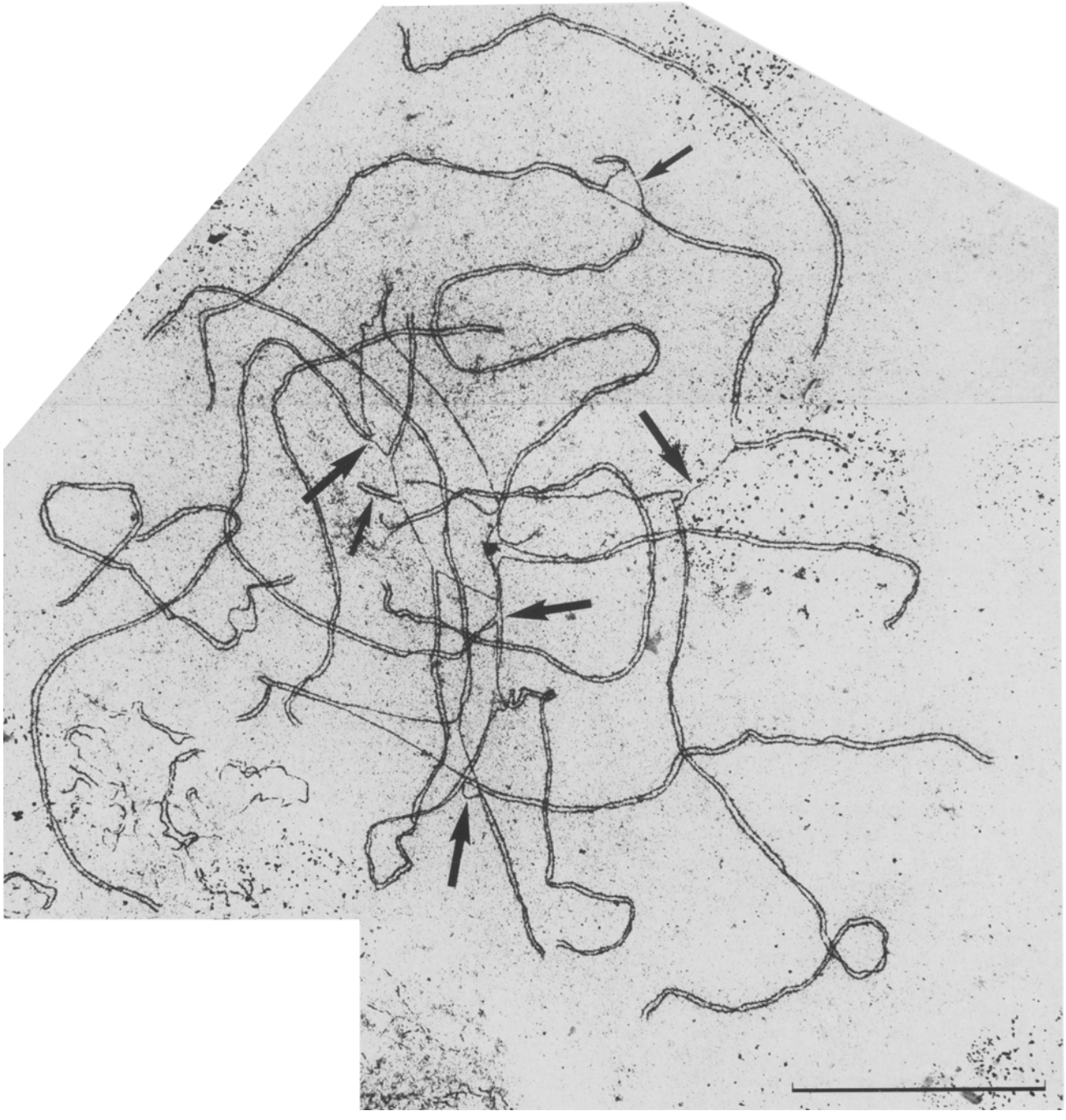

Fig. 1. Surface-spread synaptonemal complex (SC) complement of zygotene nucleus 7. Note the exchange of pairing partners (*large arrows*) and fold-back loops (*small arrows*). The fragmented SC material at bottom left is contamination from an adjacent cell. Bar represents 10 μm

which has achieved diploidisation and disomic inheritance since its formation (Raelson et al. 1989).

Materials and methods

Materials. *L. corniculatus* var. Leo (Fabaceae; $2n=4x=24$) was kindly supplied by Dr. H.G. Thomas of the Welsh Plant Breeding Station, Aberystwyth. The plants were grown one per 5 to 7 in pot in an unheated greenhouse until sampling of meiotic inflorescences in June and July 1987 and June 1989.

Methods. The scoring of pairing configurations and chiasma frequencies by light microscopy of conventional squash preparations at metaphase I of meiosis was not possible in this species because of technical difficulties in separating individual chiasmate associations at this stage. Problems of this nature have been encountered by others (Wernsman et al. 1964) and necessitated the following modifications to the usual method. Three plants bearing inflorescences at approximately the required stage were placed in a cold room at 4° C for 48 h. Inflorescences were fixed for 1 week in 3:1 ethanol:acetic acid at 4° C. Anthers from a single bud were removed and gently squashed in aceto-orcein. The anther wall debris was carefully removed, a coverslip applied, the suspension gently tapped out using the end of a fine needle and the pollen mother cells examined under light microscopy. If the cells were at diakinesis, the slide was gently heated and further squashed to facilitate scoring under a ×100 oil objective.

Whole-mount surface-spreads of synaptonemal complexes (SCs) for electron microscopy were prepared according to the methods of Albini and Jones (1984), Loidl and Jones (1986) and White and Jenkins (1987), with the following modifications. Whole anthers received a 7–10 min pretreatment in enzyme solution, prior to maceration and digestion for 10 min at 4° C. Two drops of Lipsol detergent were added to the maceration medium, left for 10 min, and washed onto plastic-coated slides with 8–10 drops of paraformaldehyde fixative.

Results

Observations of zygotene, pachytene and diakinesis are presented in chronological order to emphasise the changes in relative frequency of pairing configurations throughout meiosis. Pachytene is defined as the stage during which the *bivalents* of a nucleus are completely paired, since exchanges of pairing partners within multivalents can disrupt synapsis of chromosomes in the vicinity of the exchange. Percentage pairing and the assignment of stage, therefore, are based on the progress of pairing in bivalents only, and pairing is calculated as the total length of lateral component within SCs of bivalents expressed as a proportion of their total lateral component length. It is considered highly unlikely that asynapsis in quadrivalents interferes with the assembly of SCs in bivalents of the same complement.

Zygotene

The SCs of 13 zygotene nuclei were surface-spread. Each was analysed with respect to total lateral component length, percentage pairing and numbers of pairing configurations. These data are contained in Table 1. With the exception of nuclei 1 and 11, all the nuclei have at least one quadrivalent. A surface-spread preparation (nucleus 7) is shown in Figure 1, with its corresponding SC karyotype in Figure 2. This nucleus contains six bivalents and three quadrivalents, one of which has a double exchange of pairing partners. Other common features are irregular SCs in the form of fold-back loops and reverse pairing. These could indicate sites of structural rearrangement of the genome, but are more likely to delimit areas of non-homologous SC formation.

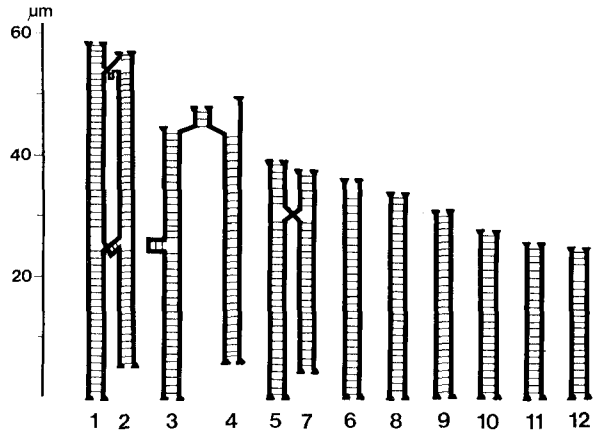

Fig. 2. SC karyotype of zygotene nucleus 7. Lateral components of the SC are represented by *vertical lines*, and the SC itself by *crosshatching*. Bivalents are numbered in decreasing order of length

Pachytene

The SCs of 24 pachytene nuclei were surface-spread and analysed in the same way as above (Table 2). A large

Table 2. Total lateral component (*LC*) length (including quadrivalents) and number of bivalents (*II*) and quadrivalents (*IV*) in the 24 pachytene nuclei

Nucleus	LC length (μm)	II	IV
1	662	8	2
2	634	10	1
3	618	10	1
4	579	8	2
5	551	10	1
6	541	10	1
7	537	12	0
8	529	10	1
9	508	10	1
10	505	12	0
11	495	12	0
12	443	10	1
13	437	10	1
14	436	12	0
15	429	10	1
16	427	10	1
17	423	10	1
18	420	10	1
19	366	12	0
20	361	12	0
21	348	12	0
22	347	12	0
23	346	10	1
24	315	12	0
Total		254	17
Mean/nucleus		10.58	0.70

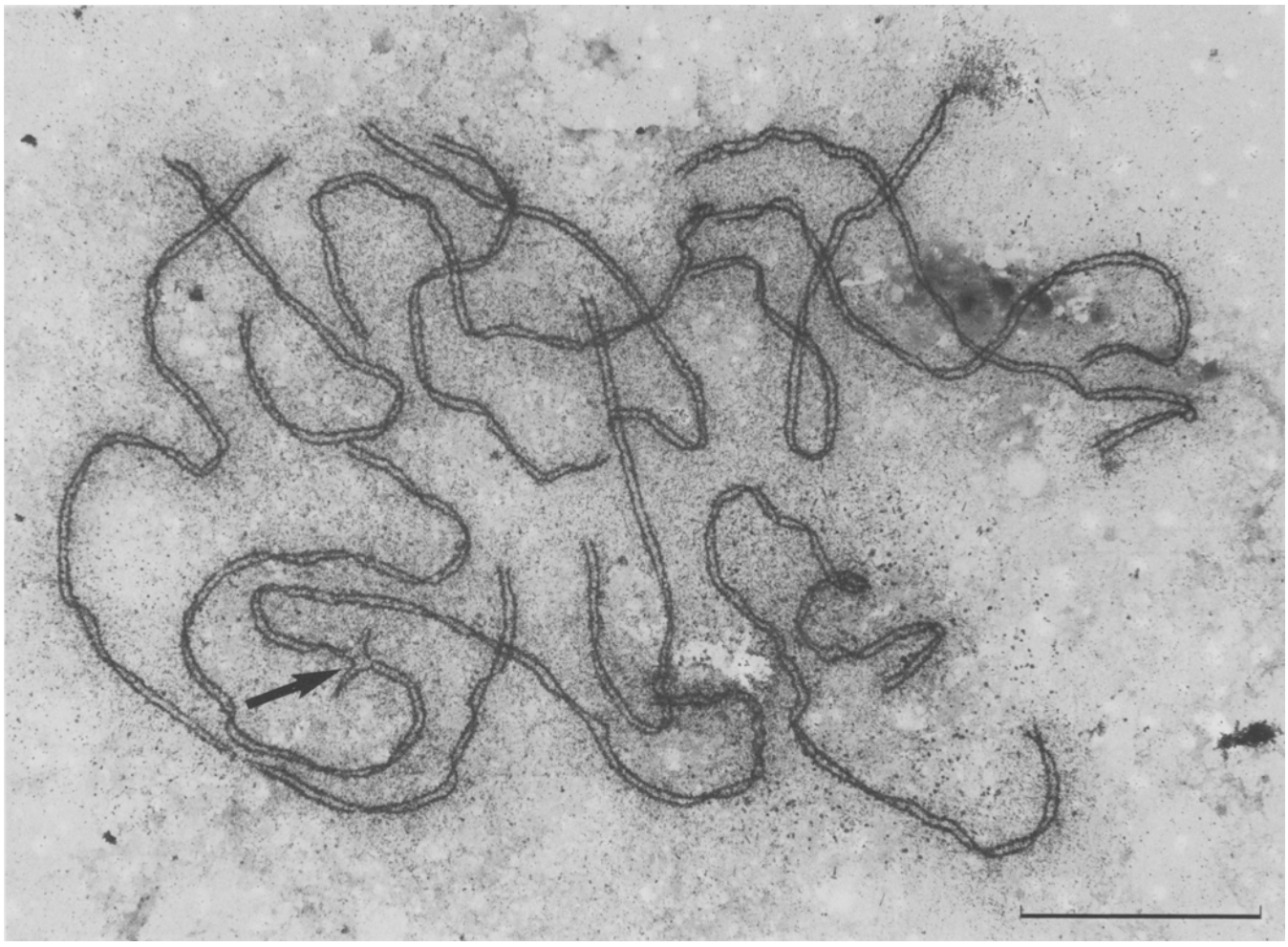

Fig. 3. Surface-spread SC complement of pachytene nucleus 18. Note the terminal exchange of pairing partners in the single quadrivalent (*arrow*). Bar represents 5 μm

proportion (37%) of the cells contain 12 bivalents and no multivalents, the first indication that there has been a shift in the relative frequencies of these pairing configurations compared with zygotene. Also, the nuclei are almost devoid of irregular non-homologous SCs in contrast to those at zygotene. Figure 3 shows a typical surface-spread at this stage (nucleus 18) with its corresponding SC karyotype in Figure 4. The nucleus comprises ten fully paired bivalents and one quadrivalent with a terminal exchange of pairing partners.

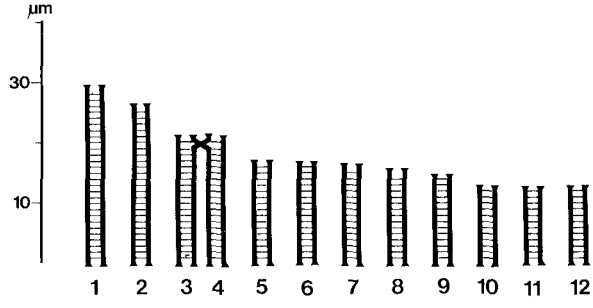

Fig. 4. SC karyotype of pachytene nucleus 18. Symbols as in Figure 2

Nature of quadrivalents

SC karyotypes similar to those in Figures 2 and 4 were constructed for each of the 37 zygotene and pachytene nuclei spread. Since centromeres were not stained by the procedures used, the actual identities of individual bivalents were not obtained. However, as an indicator of identity the bivalents of each cell were ranked in decreasing order of length and labelled 1 to 12. In the case of partially paired bivalents with a discrepancy in length between homologous lateral components, the longer of the two was taken to be the representative length of the bivalent. Table 3 records the frequency with which each ranked bivalent is involved in quadrivalent formation with other bivalents of the complement. Dispite inherent errors in identifying bivalents by length alone, particularly in a species containing some chromosomes of similar length, it can be concluded from the data in Table 3 that a substantial proportion, if not all, of the bivalents participate in quadrivalent formation. This clearly reflects the polyploid nature of *L. corniculatus* and not the presence of multiple interchanges in the population studied. It can be seen also in Table 3 that a particular chromosome pair does not form quadrivalents

Table 3. The frequencies of quadrivalent formation between the 12 ranked bivalents of the 37 zygotene and pachytene nuclei

	1	2	3	4	5	6	7	8	9	10	11	12
1	–											
2	7	–										
3	1	1	–									
4	1	0	3	–								
5	0	0	1	0	–							
6	0	2	1	2	1	–						
7	0	0	0	0	1	0	–					
8	0	0	1	0	0	0	0	–				
9	2	0	0	0	1	1	0	0	–			
10	0	0	0	0	1	0	0	1	0	–		
11	0	1	0	0	0	0	0	1	0	0	–	
12	1	0	0	0	0	0	0	1	0	0	0	–
Total	12	11	8	6	5	7	1	4	4	2	2	2

exclusively with one other bivalent, its homoeologous counterpart in the other chromosome set. To a certain extent this observation may be attributable to variation in relative length within the complement, but is unlikely to explain the association of bivalents of very different length. For example, bivalent 1 forms quadrivalents mainly with bivalent 2, its putative homoeologous partner, but also associates with bivalents 3, 4, 9 and 12. It is concluded that the latter two short bivalents at least are pairing non-homologously with bivalent 2.

Study of Table 3 also shows that the longer chromosomes participate more often in quadrivalents formation than the shorter chromosomes. This presumably reflects a greater number of pairing initiation sites consequent upon greater length, or that smaller quadrivalents are eliminated more quickly (see below).

Diakinesis

Despite technical difficulties in obtaining good squash preparations, 19 pollen mother cells (PMCs) at diakinesis were scored for pairing configurations and chiasma frequencies (Table 4). 42% of PMCs contain 12 bivalents, of which 84% are rods with one terminal chiasma. 26% of PMCs contain a single quadrivalent, and 37% up to four univalents. The univalents in all cells but one are unaccompanied by trivalents and are the result of desynapsis, since no univalency was recorded at zygotene and pachytene. The average chiasma frequency per cell of 13.6 is low, reflecting the preponderance of rod bivalents and the high frequency of univalents.

The frequency of quadrivalents recorded is much less than at zygotene and pachytene and corresponds closely to that of Wernsman et al. (1964). These quadrivalents are unlikely to be the result of an interchange, as all the plants sampled in this study and that of Wernsman et al. (1964) carried a quadrivalent. If an interchange were the cause of quadrivalent formation, some plants would be expected to be structural homozygotes. Furthermore, structural heterozygotes would be expected to show considerable infertility due to non-disjunction

Table 4. The numbers of ring and rod bivalents (*II*), quadrivalents (*IV*), trivalents (*III*) and univalents (*I*) in the 19 pollen mother cells (*PMC*s) at diakinesis

PMC	II		IV	III	I
	Ring	Rod			
1	4	7	0	0	2
2	1	9	0	0	4
3	1	11	0	0	0
4	2	8	1	0	0
5	2	10	0	0	0
6	4	6	1	0	0
7	1	11	0	0	0
8	2	10	0	0	0
9	2	10	0	0	0
10	1	11	0	0	0
11	2	10	0	0	0
12	4	6	1	0	0
13	2	9	0	0	2
14	0	11	0	0	2
15	1	11	0	0	0
16	1	8	1	0	2
17	1	9	0	1	1
18	1	9	1	0	0
19	2	9	0	0	2
Total	34	175	5	1	15
Mean/PMC	1.78	9.2	0.26	0.05	0.78

of quadrivalents at metaphase I. An estimate of pollen fertility by staining with cotton blue showed that only 3 out of 205 (1.5%) grains were infertile. This is not unequivocal proof of the absence of interchanges, since non-disjunction resulting from structural rearrangement may be tolerated in a tetraploid, or alternatively *L. corniculatus* may have evolved a mechanism for ensuring regular disjunction at metaphase I.

Elimination of multivalents

The numbers and mean frequencies of pairing configurations at zygotene, pachytene and diakinesis are reproduced for comparison in Table 5. The data for each stage were derived from a number of different plants, but since these were selected at random it was considered justifiable to pool the data for each stage. Table 5 shows that there is a reduction in mean quadrivalent frequency of 43% between zygotene and pachytene, and a greater reduction of 63% between pachytene and diakinesis. The statistical significance of the variation in frequency of

Table 5. The total numbers and mean frequencies (in *parentheses*) of bivalents (*II*), quadrivalents (*IV*), trivalents (*III*) and univalents (*I*) in the 56 nuclei at zygotene, pachytene and diakinesis

Stage	II		IV		III		I	
Zygotene	124	(9.53)	16	(1.23)	0		0	
Pachytene	254	(10.58)	17	(0.70)	0		0	
Diakinesis	209	(11.00)	5	(0.26)	1	(0.05)	15	(0.78)

Table 6. A two by three contingency chi-square analysis of the variation in number of chromosomes involved in multivalents at zygotene, pachytene and diakinesis

	Chromosomes in multivalents	Chromosomes not in multivalents	Total
Zygotene	64	248	312
Pachytene	68	508	576
Diakinesis	23	433	456
Total	155	1189	1344

$\chi^2 = 43.52$ ($P < 0.001$)

Table 7. Two by two contingency chi-square analyses of the variation in number of chromosomes involved in multivalents between zygotene and pachytene, and between pachytene and diakinesis

	Chromosomes in multivalents	Chromosomes not in multivalents	Total
Zygotene	64	248	312
Pachytene	68	508	576
Total	132	756	888
$\chi^2 = 12.12$ ($P < 0.001$)			
Pachytene	68	508	576
Diakinesis	23	433	456
Total	91	941	1032

$\chi^2 = 14.47$ ($P < 0.001$)

chromosomes involved in multivalents between the three stages of meiosis was tested by a 2×3 contingency chi-square analysis of the data (Table 6). The test confirmed highly significant ($P < 0.001$) variation in the relative numbers of chromosomes involved in multivalents at zygotene, pachytene and diakinesis. 2×2 contingency chi-square tests (Table 7) show that this variation is significant both between zygotene and pachytene ($P < 0.001$) and between pachytene and diakinesis ($P < 0.001$). Evidently, the elimination of multivalents occurs both before and after pachytene.

Discussion

The results clearly show that there are significant differences in the frequency of pairing configurations throughout meiotic prophase. The reduction in the proportion of quadrivalents from zygotene to diakinesis is not attributable to progressive abortion of cells containing quadrivalents, as there is no cytological evidence for this and abortion would not adequately explain the increase in univalent frequency. Rather, the changes in relative frequency of associations are a direct consequence of elimination of multivalents. Elimination is responsible, on average, for a reduction of 0.53 quadrivalents from zygotene to pachytene and an increase in 1.05 bivalents (Table 5). Since no trivalents or univalents are generated in this transition, it appears that the reduction in quadrivalent frequency is associated with a proportional increase in the number of bivalents only. In other words, quadrivalents are transformed into bivalents from zygotene to pachytene by the dissolution and reassembly of SCs. It was not possible to determine exactly how this was achieved. However, a likely mechanism could be the progressive dissolution of the SCs of exchanged segments and their immediate reconstitution at the site of exchange between homologous chromosomes. This could drive the sites of exchange in a quadrivalent to the ends of the chromosomes and generate bivalents. Unfortunately, it was not possible to test this hypothesis, because an insufficient number of cells was studied to monitor the kinetics of the process, and the absence of centromeres precluded the distinction between legitimately paired and exchanged segments of chromosomes.

The passage from zygotene to pachytene marks the almost complete removal of irregular, non-homologous SCs in the form of fold-back loops, reverse pairing and short associations with heterologues. Clearly, these illegitimate pairing configurations are eliminated before crossing over.

Of the multivalents persisting to pachytene, a very large proportion (over 60%) are not consolidated by chiasma formation. Presumably some of these are resolved to bivalents before crossing over by a mechanism similar to that outlined above. Others probably persist through the period of genetic exchange, but fail to form chiasmata in synapsed segments which are not strictly homologously paired. The failure of chiasma formation could be the result of interference by the ongoing elimination process itself of crossing over in exchange segments, or an active suppression of the crossover machinery in homoeologous and non-homologous SCs, as has been shown in wheat (Holm and Wang 1988) and ryegrass hybrids (Jenkins 1988). It is possible, of course, that the low chiasma frequency of this organism is not the result of elimination but the cause of it. In other words, a dearth of chiasmata inevitably results in a reduction in quadrivalent frequency at diakinesis. Indeed, the high frequency of rod bivalents at diakinesis (Table 4) may be a manifestation of failure of crossing over in the two opposite arms of some quadrivalents.

The transition from pachytene to diakinesis also marks the appearance of univalents. These are probably generated upon entry into diplotene from pairing configurations which are not consolidated by chiasmata. This implies that there may be bivalents at pachytene that comprise homoeologous or non-homologous chromosomes. These could be formed from the beginning of synapsis or generated de novo by an elimination mechanism which can transform quadrivalents to bivalents, but cannot discriminate between homologous and homoeologous chromosomes. This may be because *L. corniculatus* is a relatively recent tetraploid species and is in the process of evolving a more efficient pairing control system analogous to that in wheat (Riley and Chapman 1958; Okamoto 1957). Diploidisation in wheat is governed by genes mainly at the Ph locus, which ensure that most multivalents formed at zygotene are resolved

into homologous bivalents before crossing over (Hobolth 1981; Holm 1986; Holm and Wang 1988). In addition, *L. corniculatus* may be a segmental allotetraploid containing four very similar sets of chromosomes. This could confound the discrimination between homologous and homoeologous chromosomes and reduce the overall efficiency of diploidisation.

In conclusion, the results demonstrate that diploidisation is the product of several processes operating at different times throughout meiotic prophase. Some homologous bivalents are formed from the onset of synapsis, while others are the result of elimination of multivalents before pachytene. Crossing over presents special problems, as elimination mechanisms acting subsequently rely on precise placement of crossovers in homologously paired regions only, or suppression of crossovers in illegitimately synapsed segments. Diploidisation is dependent on the efficiency of each of these constituent processes, the relative importance and precision of which varies between species.

Unfortunately, this study of chromosome pairing behaviour during meiotic prophase has added little insight into the origin of this species. The extent of SC formation cannot be used as a gauge of the relatedness of the two genomes of this tetraploid, since it is likely that a proportion at least of SC is actually non-homologous. Furthermore, the action of Ph-like genes could modify chromosome associations formed solely on the basis of their genetic or structural similarity.

Acknowledgements. The authors acknowledge with gratitude financial support from the Agricultural and Food Research Council (Grant no. PG2/123), and GJ thanks the Royal Society for continuing support.

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