Theor Appl Genet (2001) 103:219–230 © Springer-Verlag 2001

K.-B. Lim · M.S. Ramanna · J.H. de Jong
E. Jacobsen · J. M. van Tuyl

Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH

Received: 15 May 2000 / Accepted: 4 December 2000

Abstract A detailed analysis of microsporogenesis was carried out in three diploid lily cultivars (2n=2x=24) and three diploid interspecific hybrids (2n=2x=24) using DNA *in situ* hybridisation methods (GISH and FISH). In cvs. Gelria (*Lilium longiflorum*; L genome), Connecticut King and Mont Blanc (both Asiatic hybrids; A genome) meiosis was regular and only haploid gametes were formed while the three interspecific hybrids between *L. longiflorum*×Asiatic hybrid (LA) showed a variable frequency of meiotic nuclear restitution and stainable 2n-pollen formation ranging from 3% to 30%. An analysis of meiotic chromosome behaviour of the LA hybrids through GISH and FISH revealed that: (1) the parental chromosomes could be clearly discriminated into univalents, half-bivalents and bivalents in the PMCs; (2) in some of the PMCs the entire complement was present either as univalents or half-bivalents which had the potential to divide equationally (following centromere division) during the first division leading to first division restitution (FDR) gametes; (3) more frequently, however, in one and the same PMC the univalents and half-bivalents divided equationally whereas the bivalents disjoined reductionally at the same time giving rise to 2n-gametes that could vary from the well-known FDR or SDR 2n-gametes. We indicate this novel type of restitution mechanism as Indeterminate Meiotic Restitution (IMR). In order to confirm the occurrence of IMR gametes, the chromosome constitutions of eight triploid BC_1 progenies derived from backcrossing the 2n-gamete producing the LA hybrids to the Asiatic hybrid parents were analysed through *in situ* hybridisation. The results indicated that there were seven BC_1 plants in which FDR 2n-gametes, with or without homoeologous recombinations, were functional, whereas in one case the 2n-gamete resulting from IMR was functional. In the latter, there was evidence for the occurrence of genetic recombination through homoeologous crossing-over as well as through the assortment of homoeologous chromosomes. A singular feature of the IMR 2n-gamete was that although it transmitted a euploid number of 24 chromosomes to the BC_1 progeny, the number of chromosomes transmitted from the two parental species was dissimilar: 9 L-genome chromosomes and 15 A-genome chromosomes instead of 12 of each.

Keywords *Lilium* · Restitution mechanism · Meiotic polyploidisation · Indeterminate meiotic restitution (IMR) · First division restitution (FDR) · Second division restitution (SDR) · Fluorescence *in situ* hybridisation (FISH)

Communicated by B.S. Gill

K.-B.Lim · J.M. van Tuyl (✉)
Plant Research International,
Business Unit Genetics and Breeding, P.O. Box 16,
6700 AA Wageningen, The Netherlands
e-mail: j.m.vantuyl@plant.wag-ur.nl
Fax: +31-317-418094

M.S. Ramanna · E. Jacobsen
Laboratory of Plant Breeding, Wageningen University,
Wageningen, The Netherlands

J.H. de Jong
Laboratory of Genetics, Wageningen University, Wageningen,
The Netherlands

Introduction

It has been estimated that more than 70% of the flowering plants are polyploids (Soltis and Soltis 1993; Masterson 1994; Leitch and Bennett 1997; Ramsey and Schemske 1998) including many important crops such as wheat (*Triticum aestivum* L.), potato (*Solanum tuberosum* L.), cotton (*Gossipium hirsutum*, *G. barbedense*) and sugarcane (*Saccharum officinarum* L.). Among horticultural crops, spontaneous polyploids have contributed greatly to the development of cultivars. Thus, polyploidy is an important phenomenon both for evolution as well as in the development of new cultivars. Despite the appreciation of the importance of polyploidy in plants, little attention has been paid to their modes of origin. The early belief that polyploids originated from chromo-

some doubling of somatic cells (mitotic polyploidisation) was seriously questioned by Harlan and De Wet (1975). They argued that numerically unreduced (2n) gametes have played a predominant role in the origin of polyploids. Meiotic abnormality that leads to the formation of 2n gametes, the so-called meiotic nuclear restitution, was described for the first time by Rosenberg (1927). Subsequently, various types of meiotic nuclear restitution mechanisms, such as semiheterotypic division, pseudohomoeotypic division, mitotised meiosis, first division restitution (FDR), second division restitution (SDR), and pre- and post-meiotic doubling have been described in various plant species (for reviews, see Ramanna 1979; Veilleux 1985). From several investigations, it is clear that meiotic nuclear restitution leading to 2n-gamete formation can occur in normal fertile plants as well as in wide interspecific hybrids that are otherwise sterile.

In *Lilium* (2n=2x=24), as in most other taxa, wide interspecific hybrids are, in general, sterile. This is especially true for hybrids of species belonging to different taxonomic sections. Sterility in these hybrids mainly results from irregular chromosome associations between the parental genomes during meiosis (Asano 1982, 1984). When completely sterile, the interspecific hybrids cannot be used for further cross breeding. One way of restoring the fertility of sterile diploid lily hybrids has been to double the chromosome number by chemical treatment (Van Tuyl et al. 1992). A major drawback of this approach is that, due to preferential chromosome association in the amphidiploid, intergenomic recombination by crossing-over is extremely decreased (Lim et al. 2000). This approach is obviously not favourable for introgression breeding.

Fortunately, certain interspecific lily hybrids produce relatively high frequencies of 2n-gametes (Asano 1984; Van Tuyl et al. 1989). Such hybrids offer the prospect of using diploid hybrid genotypes directly for introgression breeding without the need for mitotic chromosome doubling. In lily, this possibility has already been demonstrated in the chromosome analysis of some progenies derived from the functioning of 2n-gametes (Karlov et al. 1999). In this report, we show that two types of FDR gametes were functional in the origin of BC_1 progenies of lilies. These included FDR gametes with and without homoeologous recombinant chromosomes.

Although both FDR and SDR gametes have been shown to occur in some plant species, the cytological evidence is still rare. Half-tetrad analysis (Mok and Peloquin 1975) and genetic or molecular markers (Bastiaanssen et al. 1999) have been used to elucidate the restitution mechanisms. FDR 2n-gametes comprise the non-sister chromatids of each homoeologous chromosomes in an interspecific hybrid resulting in 12 chromosomes from each parent. However, SDR 2n-gametes contain both sister chromatids from one parent, whereas the homoeologous sister chromatids will be included in the counterpart 2n-gamete within a dyad (if the composition of the parental chromosomes of one gamete is 4A+8B then the counterpart should be 8A+4B).

In an attempt to elucidate the restitution mechanisms in *Lilium* interspecific hybrids and to assess their consequences in the progenies, we used *in situ* hybridisation techniques for the analysis of microsporogenesis in the hybrids and for constructing the karyotypes of the BC_1 progenies. This study has led to the discovery of a new mechanism of restitution gamete formation. This mechanism (indeterminate meiotic restitution: IMR) has been illustrated and discussed in relation to other results.

Material and methods

Plant material

Three diploid (2n=2x=24) 2n-gametes producing interspecific hybrids of *Lilium longiflorum* (L) Gelria×Asiatic hybrid (A) Whilito as well as their backcross progenies were used for genomic *in situ* hybridisation (GISH) analysis. The hybrids were produced through integrated pollination and embryo rescue methods (Van Tuyl et al. 1991). The detailed information of these F_1 hybrids (LA_1–LA_3) and BC_1 plants (ALA_1–ALA_8) is given in Table 1. All F_1 hybrids were known to produce viable 2n-pollen at levels ranging from about 1% up to 12%. In order to produce backcross progenies we used, four different Asiatic hybrids (all diploid) as female parents and crossed these to the three above-mentioned LA hybrids. Among the four female parents, three were cultivars (Montreux, Puccini and Meribel) and one genotype was a breeding parent (78251). Plants were grown in a greenhouse at 20°–25°C during the day and 14°–18°C during the night.

Pollen germination test

Mature pollen was placed on the artificial agar medium containing 100 g sucrose, 5 g agar and 20 mg boric acid per litre. After a 12-h culture at 20°C the germinated pollen was counted and classified as large (2n) or small (n).

Chromosome preparation

For the analysis of microsporogenesis and mitotic chromosome constitution, young anthers with prophase I through telophase II stages were fixed in a freshly prepared solution of 3:1 (v/v) ethanol:acetic acid for 1–2 h at room temperature. Parts of fixed anthers were squashed in a drop of 2% acetocarmine to determine

Table 1 Origin and parentage of interspecific hybrids and their backcross progeny

	Genome type[a]	Accession number	Female parent×male parent
F_1 hybrids	$LA_{1\sim3}$	88542-24, 52, 69	*L. longiflorum* Gelria×Asiatic hybrid Whilito
BC_1 progeny	ALA_1	901122-1	Asiatic hybrid 78251×88542-24
	$ALA_{2\sim4}$	921238-1, 2, 3	Asiatic hybrid Montreux×88542-52
	$ALA_{5\sim6}$	985099-1, 2	Asiatic hybrid Puccini×88542-52
	$ALA_{7\sim8}$	942460-1, 2	Asiatic hybrid Meribel×88542-69

[a] L and A, *L. longiflorum* and Asiatic hybrid, respectively

appropriate meiotic stage, and slides with good cells were prepared for GISH analysis. In order to study the process of cytokinesis and the sporad stages, we mounted pollen mother cells (PMCs) in a drop of lactophenol-acid fuchsin. Similarly, pollen from fully opened flowers was used for stainability and size classification as small (n) and large (2n). Pollen size was determined using a calibrated micrometer.

For the somatic chromosome preparation, root tips were collected in the early morning, pre-treated in a saturated α-bromonaphtalene solution and kept at 4°C until the next morning for accumulation of metaphase cells. Subsequently the root tips were fixed in the ethanol – acetic acid solution (3:1) for at least 2 h and stored at –20°C until use.

Anthers or root tips were incubated in a pectolytic enzyme mixture containing 0.3% pectolyase Y23, 0.3% cellulase RS and 0.3% cytohelicase in 10 m*M* citrate buffer (pH 4.5) at 37°C for about 1–1.5 h. Preparations were squashed in a drop of 60% acetic acid and frozen in liquid nitrogen; the cover slips were removed by using a razor blade. Slides were dehydrated in absolute ethanol for a few minutes, dried and stored at –20°C until use.

Probes DNA preparation

DNA probes consisted of either genomic DNA from *L. longiflorum* or a rDNA sequence from pTa 71 (45 S rDNA). Clone pTa71 contains the 9 kb *Eco*RI fragment of 45 S ribosomal DNA from wheat (Gerlach and Bedbrook 1979). The *L. longiflorum* DNA and 45 S rDNA sequence were labelled with digoxigenin-11-dUTP and biotin-16-dUTP by nick translation.

Fluorescence *in situ* hybridisation (FISH)

Slides with chromosome complements were left overnight at 37°C. The next day the slides were treated with 100 µg/mL RNase A in 2× SSC at 37°C for 60 min and washed three times with 2× SSC at room temperature for 5 min. The slides were then incubated with 10 m*M* HCl at 37°C for 2 min, treated with 100 µl of a pepsin solution (5 µg/ml in 10 mM HCl) at 37°C for 10 min, followed by washing twice in 2× SSC for 5 min, then with 4% (para) formaldehyde solution for 10 min and finally washed in 2× SSC three times, 5 min each. Before air-drying, the slides were rapidly dehydrated in a graded alcohol solution series (70%, 90%, 100%) for 3 min each. Samples of 40 µl of the hybridisation mixture containing 100 ng of the probe DNA, 2 mg of sheared herring sperm DNA (GIBCO BRL), 50% deionised formamide, 10% (w/v) sodium dextran sulphate (Sigma), 2× SSC and 0.25% (w/v) SDS were denatured for 5 min at 70°C and then directly put on ice for at least 5 min. Each slide with 40 µl of the hybridisation mix and covered with a slip of plastic sheet was denatured for 5 min at 80°C and left overnight at 37°C in a tightly closed humidified container. The slide was then washed in 2× SSC buffer for 15 min, transferred to 0.1× SSC buffer at 42°C for 30 min and incubated at 37°C for 60 min in blocking buffer [0.1 *M* maleic acid, 0.15 *M* NaCl, 1% (w/v) blocking reagent from Boehringer Mannheim]. Biotin-and/or digoxigenin-labelled probe DNA was detected using the Cy3-Avidin-streptavidin detection system (Vector Laboratories) and/or the FITC-anti-digoxigenin detection system (Boehringer Mannheim, Germany), respectively. All slides were counterstained either with 10 mg/ml DAPI (4′,6-diaminido-2-phenylindole) or with 5 mg/ml propidium iodide (PI). Images were photographed with a Zeiss Axiophot photomicroscope equipped with epi-fluorescence illumination and single-band filters for DAPI, FITC and Cy3/PI using 400 ISO colour negative film. The film was then scanned at 1200 dpi for digital processing with the image software PHOTOSHOP (version 5.0; Adobe).

Chromosome numbering

A revised method of nomenclature of *Lilium* chromosomes has been proposed by Lim et al. (2000) in which the chromosomes are numbered from **1** for the longest short-arm chromosome to 12 for the chromosome with the shortest short arm in the karyotype. The genomes of the species are indicated by L and A followed by the individual chromosome number of the *L. longiflorum* and the Asiatic hybrids, respectively.

Results

Meiotic chromosome analysis

Microsporogenesis was analysed in the three diploid cultivars (2n=2x=24) Gelria, Connecticut King and Mont Blanc and in the three diploid LA hybrids. In all three cultivars, chromosome association was normal with the formation of 12 bivalents (data not shown). Following this, anaphase I disjunction was normal, and the most notable feature was that at the end of telophase I the resulting nuclei were separated by the formation of a cell wall, the so-called reductional wall. After the pro-, meta- and anaphase II stages, the equational wall was formed at telophase II giving rise to a tetrad. Thus, in *Lilium*, microsporogenesis is of the "successive type" as far as cell-wall formation is concerned.

Abnormal meiosis was observed in all three LA hybrids with regard to chromosome association at anaphase I. In many cases, an unbalanced anaphase I disjunction of the chromosomes was observed, leading to the absence of the second meiotic division. At metaphase I, there was clear evidence for homoeologous chromosome association (Fig. 1a). The frequency of chromosome association varied in different PMCs ($24_I/0_{II}$–$6_I/9_{II}$). One of the difficulties in estimating the frequency of univalents and bivalents at metaphase I was that meiosis was highly asynchronous within each anther. Because of this, some of the bivalents were precociously disjoined, thereby contributing to an underestimation of the frequency of bivalents. Precocious separation of bivalents was evident from the presence of half-bivalents (Fig. 1b) in addition to univalents at metaphase I. Due to the presence of recombinant segment(s) in the chromatid(s), half-bivalents could be distinguished clearly from the univalents. How-

Table 2 Chromosome associations at late-metaphase I of the three 2n-gametes producing interspecific hybrids (2n=2x=24). (*PMCs* pollen mother cells)

Genome and accession		Number of PMCs analysed	Chromosome configuration (%)	
			Univalent	Bivalent
LA_1	88542-24	30	14.8 (62)	4.6 (38)
LA_2	88542-52	103	19.6 (81)	2.2 (19)
LA_3	88542-69	33	17.2 (72)	3.4 (28)

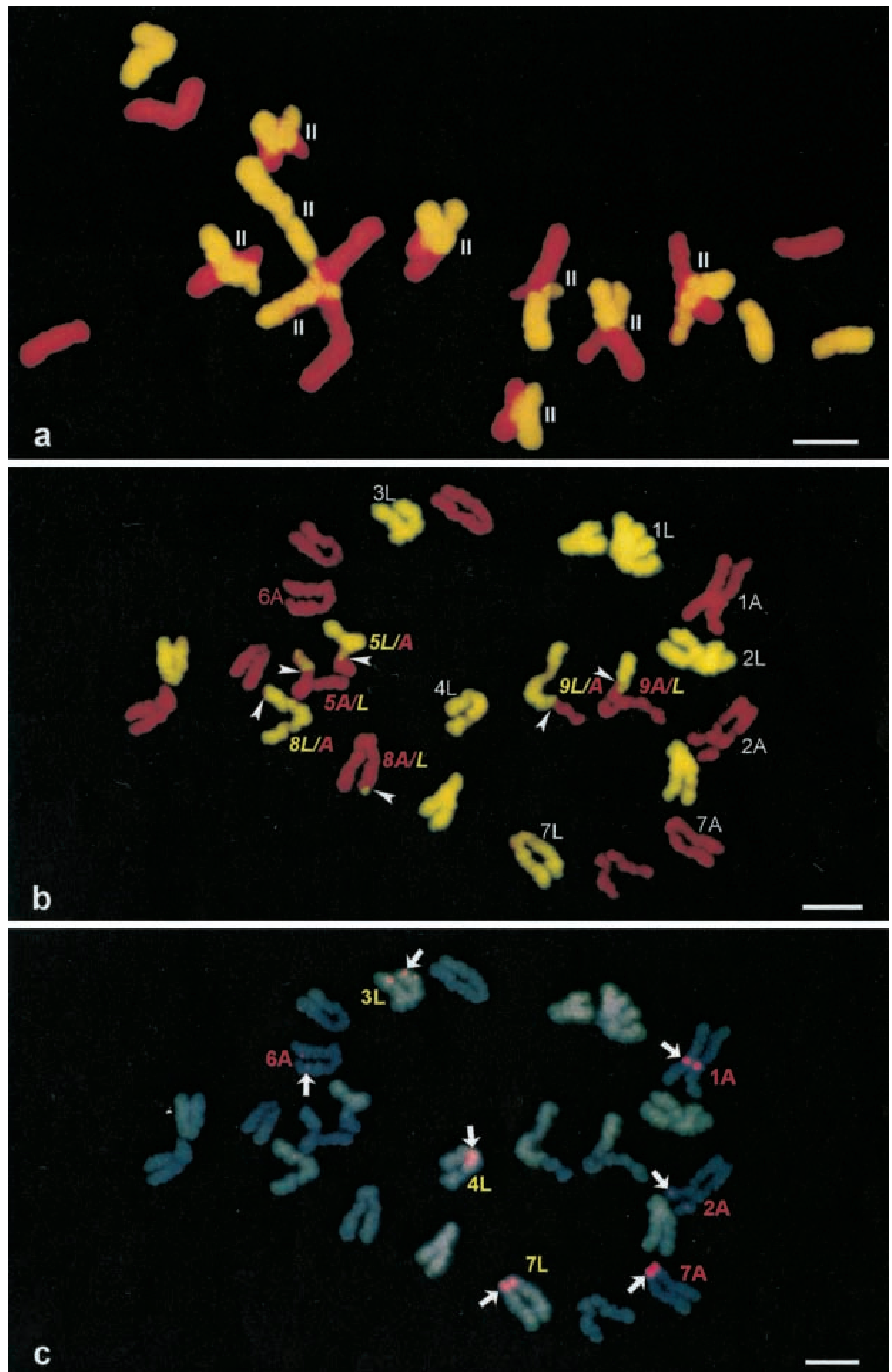

Fig. 1a–c Early- and late-metaphase I in the pollen mother cells of the F_1 plants of *Lilium longiflorum*×Asiatic hybrid (LA). In **a** and **b** the chromosomes are hybridised with total genomic DNA of *L. longiflorum* detected with anti-dig FITC (*yellow*) and counterstained with propidium iodide (Asiatic genome being *red*). **a** Metaphase I showing $9_{II}+6_{I}$. On the *left*, a pair of bivalents with homoeologous recombinations. Note: the number of univalents corresponds with the expectation – three of *L. longiflorum* (*yellow*) and three of Asiatic (*red*). **b** A late-metaphase I showing univalents and half-bivalents, corresponding to the expected 24 bodies. Some of the univalents and all the half-bivalents, which could be identified, are labelled appropriately. The half-bivalents are indicated with the respective chromosome (centromere) followed by the homoeologous chromosome that contributed the recombinant segment. For example, 9L/A-9A/L (in their respective colours). *Numbers* and *letters* in *italics* indicate half-bivalents according to suspected chromosome number based on the length of the short arm. Recombination break-points are shown by *arrowheads*. **c** The same cell as in **b** but hybridised with the 45 S rDNA probe (pTa 71). Note the distinct hybridisation sites, which serve as markers for
the identification of seven different chromosomes in the LA hybrid – three chromosomes of the L-genome and four chromosomes of the A-genome. *Bar* (**a**–**c**) 10 μm

ever, it was likely that some of the half-bivalents with very small recombinant segments might have escaped GISH detection. Table 2 shows the frequency of chromosome association at metaphase I of the LA hybrids. A relatively high frequency of chromosome association was observed, ranging from $19.5_{I}/2.2_{II}$–$14.8_{I}/4.6_{II}$ of PMCs among the three LA hybrids (Table 2). A unique feature in the three LA hybrids was that the process appeared to be delayed in some of the PMCs with sporad stages occurring alongside chromosomal division stages. Invariably, the univalents and half-bivalents in PMCs with delayed division appeared more like somatic chro-

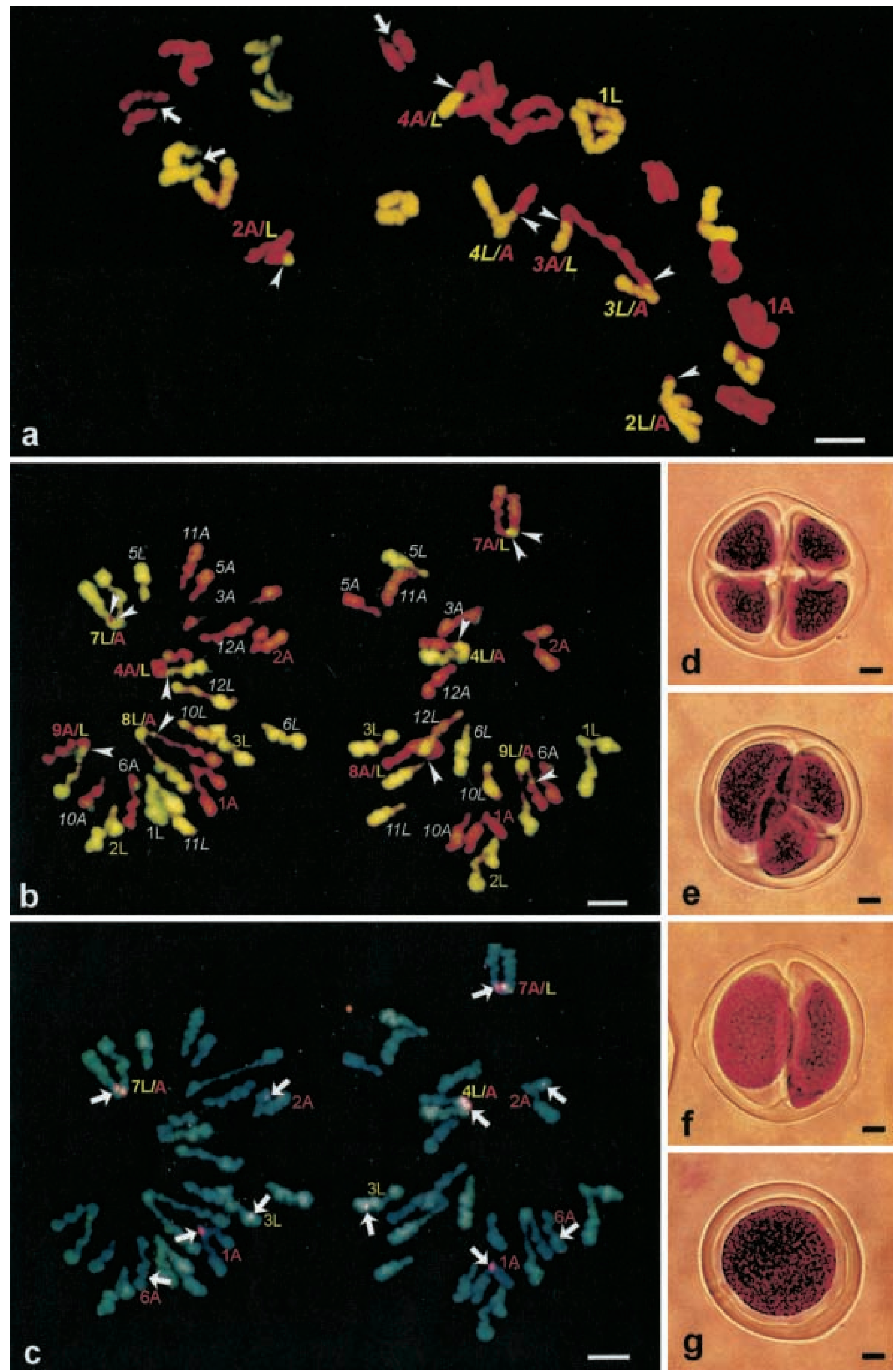

Fig. 2a–g "Division" of chromosomes in the stage following late-metaphase I and sporad formation in the LA hybrid. **a** Late-meta/early-anaphase I showing "division" of bivalents and univalents. Note: some of the bivalents, which have already disjoined, are represented by two half-bivalents, i.e. 2A/L-2L/A, 3A/L-3L/A and 4A/L-4L/A. The centromeres of the univalents indicated by *arrows* have already divided equationally. *Arrowheads* indicate the breakpoint of the half-bivalents. The labels of some of the chromosomes have been given the corresponding chromosome number. **b** Distribution and division of half-bivalents and univalents following late-metaphase I. In this modified anaphase stage, equational division of the univalents as well as disjunctional separation of the half-bivalents were confirmed through GISH. Some of the half-bivalents that could be identified based on 2c are marked, e.g. 4A/L-4L/A and **7** A/L-7L/A. All of the univalents that divided equationally are also identified with *numbers* and *letters*. *Numbers* combined with a *letter* in *normal print* represent the exact chromosome number of both genomes. *Numbers* and *letters* in *italics* indicate a doubtful identification. Nevertheless, based on their positioning in the two poles the numbers are helpful for the placement of the respective counterparts of the dyad. **c** The same cell as in **b** but reprobed with 45 S rDNA sequences (pTa 71) to identify the counterpart chromosomes at both poles. The presence of half-bivalents at the poles was confirmed for 7A/L-7L/A and 4L/A based on FISH detection. Similarly, the equational division of univalents was also confirmed for 1A, 2A, 3L and 6A. **d**–**g** Sporad stages showing a tetrad (**d**), triad (**e**), dyad (**f**) and monad (**g**). *Bar* (**a**–**g**): 10 μm

mosomes than meiotic chromosomes (compare Fig. 1b with Fig. 1a).

Because the positions of the centromeres of both the L and A genomes (yellow and red, respectively, in Figs. 1 and 2) were clearly determined for many of the chromosomes, univalents and half-bivalents could be identified (marked with L, A, L/A, A/L in Fig. 1b). For further identification of those chromosomes, FISH detection of 45 S rDNA sites with probe pTa71 was employed (Fig. 1c). Four chromosomes of the A genome (chromosome nos. 1, 2, 6 and 7) and three of the L-genome (chromosome nos. 3, 4 and 7) possessed 45 S rDNA sites. These served as markers for the identification of nucleolar organising regions (NORs) bearing univalent and half-bivalent chromosomes (see Figs. 1c and 2c). The salient features of the behaviour of univalents and

Table 3 Number (percentages) of different types of sporads in LA hybrids

Accession	PMCs					
	Monad	Dyad	Triad	Tetrad	Others	Total
LA_1 88542-24	40 (17)	58 (25)	41 (17)	86 (36)	11 (5)	236 (100)
LA_2 88542-52	48 (20)	99 (40)	16 (7)	68 (28)	12 (5)	243 (100)
LA_3 88542-69	2 (1)	46 (11)	70 (17)	281 (66)	19 (5)	418 (100)

half-bivalents in later meiotic stages (Fig. 1a for metaphase I; Figs. 1b and 2a for late metaphase I; Fig. 2b for modified anaphase stage) were that:

1) the chromatids and the centromeres were more pronounced than in earlier stages (Fig. 1b, compare to Fig. 1a);
2) most of the univalents, half-bivalents and bivalents were oriented on the equatorial plate of the cell (Fig. 2a);
3) all univalents divided equationally through centromere division (Fig. 2b);
4) bivalents (when present) disjoined reductionally in all cases (Fig. 2b).

The occurrence of the aforementioned chromosome features was observed in all of the LA hybrids. The result was that two groups of chromatids that led to a disproportionate number of chromosomes of the genomes (deviating from the expected 12+12) could be found in modified anaphase stages (Fig. 2b). The chromosomal orientation and equational division represent a modified form of meiosis where only one division of chromosomes occurs. The successive cytokinesis that was normally expected to occur after telophase I in these cells was totally absent. An important feature was that it was possible to determine the distribution of the chromatids to the two poles during the anaphase. This observation was obvious from GISH and FISH. The simultaneous reductional separation of bivalents and equational division of univalents could be detected in modified anaphase stages (Fig. 2a, b). This phenomenon was also confirmed by FISH for those chromosomes containing 45 S rDNA sites (Fig. 2c). Thus, the division of univalents could be discriminated from those of half-bivalents. In the case of univalents the division was strictly equational, but the half-bivalents divided in two alternative ways:

1) two non-recombinant or two recombinant chromatids of a pair of half-bivalents (two half-bivalents; they were one bivalent in metaphase I) moved to each pole (figure not shown)
2) a non-recombinant and a recombinant chromatid of a pair of half-bivalents moved to opposite poles (for example, 7L/A and 7A/L in Fig. 2b).

There was also evidence for the inclusion of two sister chromatids of a half-bivalent at the same pole (for example, chromosomes nos. 4, 7, 8 and 9 in Fig. 2b). In order to summarise the consequences of these abnormal chromosomal distributions with respect to the composition of 2n-gametes, the possible mechanisms of meiotic restitution are schematically illustrated in Fig. 3.

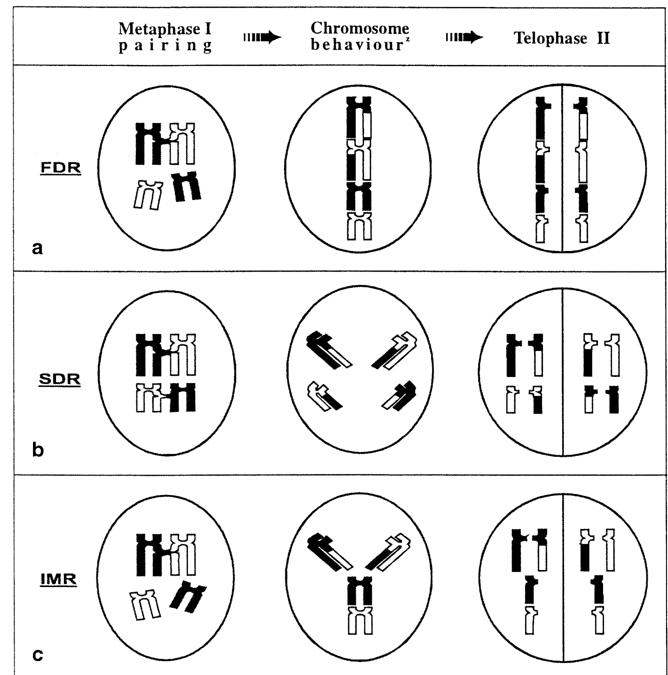

Fig. 3a–c A schematic representation of three possible types of meiotic nuclear restitution in a diploid interspecific hybrid in the case of 2n=2x=4. The homoeologous pairs of chromosomes are shown as *black* and *white* chromosomes. **a** First division restitution (FDR) with recombination. At metaphase I, one bivalent and two univalents are formed. In the subsequent stage two half-bivalents and two univalents align on the equatorial plate and divide equationally. The result is that the homoeologous chromosomes do not assort independently and that the centromeres of both genomes are intact in the 2n-gametes. **b** Second division restitution (SDR) with recombination showing independent assortment of homoeologous pairs of chromosomes. In this case both pairs of homoeologous chromosomes disjoin at anaphase I but restitute subsequently, i.e. without the second division. The notable features of SDR are that the homoeologous pairs assort independently of each other and that the number of centromeres of the parental genomes are not preserved intact in the resulting 2n-gametes. Moreover, each centromere is always represented in pairs. **c** Indeterminate meiotic restitution (IMR) showing unequal distribution of the centromeres of the parental genomes. At metaphase I a bivalent and two univalents are formed. The bivalent disjoins normally as in the anaphase I, whereas the two univalents divide equationally. Consequently, the chromosome constitution of the parental genomes is not preserved in the 2n-gametes and, furthermore, the centromeres of each of the parental genomes are present in odd numbers. In all three cases (**a–c**), meiosis is incomplete. Because of this, the different stages of meiosis cannot be strictly defined

Cytokinesis and pollen formation

Based on the analysis of sporads (Table 3) it was possible to determine the type of cytokinesis that had occurred in each PMC. Accordingly, two equational walls

Table 4 Percentage (%) of stainable, large and small pollen grains and germination in six different genotypes of *Lilium*

Genome, cultivar or accession	Total	Number (%) of stainable pollen counted				Germination[c] (%)
		Stained pollen[a]	Large (2n)	Small (n)	Size (μm)[b]	
L genome, Gelria	1187	761 (64)	0 (0)	761 (64)	89±7	54
A genome, Connecticut King	942	610 (65)	1 (0)	609 (65)	59±6	53
A genome, Mont Blanc	1021	883 (86)	2 (0)	881 (86)	56±4	60
LA hybrids, 88542-24	1403	58 (4)	45 (3)	13 (1)	98±9	<1
88542-52	568	227 (40)	171 (30)	57 (10)	102±13	12
88542-69	838	80 (10)	62 (7)	18 (2)	106±15	8

[a] Pollen stained by lacto-phenol acid fuchsin
[b, c] Pollen size and germination (%) of the LA hybrids indicate 2n-gametes only

Table 5 Number of chromosomes and chromosome constitution of interspecific hybrids and their backcross progeny

	Genome type[a]	Somatic chromosome number (2n)	Chromosome constitution[a]		Number of cross-over break-points	Number of recombinant chromosomes			Mechanism of the 2n-gamete involved
			L	A		L/A[b]	A/L[b]	Total	
F_1	LA_1	24	12	12	0	0	0	0	–
	LA_2	24	12	12	0	0	0	0	–
	LA_3	24	12	12	0	0	0	0	–
BC_1	ALA_1	36	9	27	10	1	4	5	IMR
	ALA_2	36	12	24	7	2	1	3	FDR
	ALA_3	36	12	24	0	0	0	0	FDR
	ALA_4	48	12	36	0	0	0	0	FDR
	ALA_5	36	12	24	9	3	4	7	FDR
	ALA_6	48	12	36	7	5	0	5	FDR
	ALA_7	36	12	24	1	0	1	1	FDR
	ALA_8	36	12	24	0	0	0	0	FDR

[a] L and A, *L. longiflorum* and Asiatic hybrid, respectively
[b] L/A and A/L, Recombinant chromosomes of *L. longiflorum* chromosome (centromere) with Asiatic chromosome segment(s) and Asiatic hybrid chromosomeS (centromere) with *L. longiflorum* chromosome segment(s); respectively

(following anaphase II) formed in two compartments of a PMC, giving rise to a tetrad. In all three LA genotypes varying numbers of tetrads were formed from 28% to 66% (Table 3). Obviously, the four spores were unbalanced, leading to sterility. There was, however, a considerable percentage of triads (7–17%) that indicated the occurrence of a reductional wall and of an equational wall in one of the cells (Table 3 Fig. 2e). In the rare event of normal anaphase I separation, the presence of triads indicated the potential for second division restitution (SDR). The dyad was the other most abundant class, varying in frequency from 11% to 40%. In this case, considering the chromosomal divisions in PMCs with delayed meiosis, dyad formation was due to the formation of one equational wall. The high frequency of dyad formation in genotype, 88542–52 was in agreement with the higher frequency of 2n-pollen recorded (Tables 3, 4).

In view of the regular meiotic divisions in all the three diploid cultivars, pollen fertility was expected to be high, as shown in Table 4. In the three LA hybrids, pollen stainability was lower, i.e. 4%, 40% and 10%. Among these, the large pollen grains, representing 2n-pollen, were in the order of 3%, 30% and 7% respectively. Upon germination on artificial medium, pollen tubes emerged with frequencies of less than 1%, 12% and 8%, corresponding to the frequencies of 2n-pollen (Table 4).

Chromosome constitution of the backcross progenies (BC_1)

In order to determine whether the 2n-gametes had originated through IMR, the chromosome constitutions of eight BC_1 plants were analysed through *in situ* hybridisation. All eight plants had originated through the functioning of 2n-gametes from the LA hybrid and n- or 2n-gametes from the backcross parent, an Asiatic hybrid. Thus, these eight plants were either triploids (2n=3x=36) or tetraploids (2n=4x=48); their chromosome constitution and the number of homoeologous crossing-overs in each case are given in Table 5.

The ALA_1, a triploid BC_1 plant, possessed 27 A- and only nine L-chromosomes (Fig. 4a). Despite the disproportionate number of chromosomes from the L- and A-genomes, this plant possessed 36 chromosomes as if all the three complete sets of the chromosome were present. Detection of the 45 S rDNA probe as diagnostic

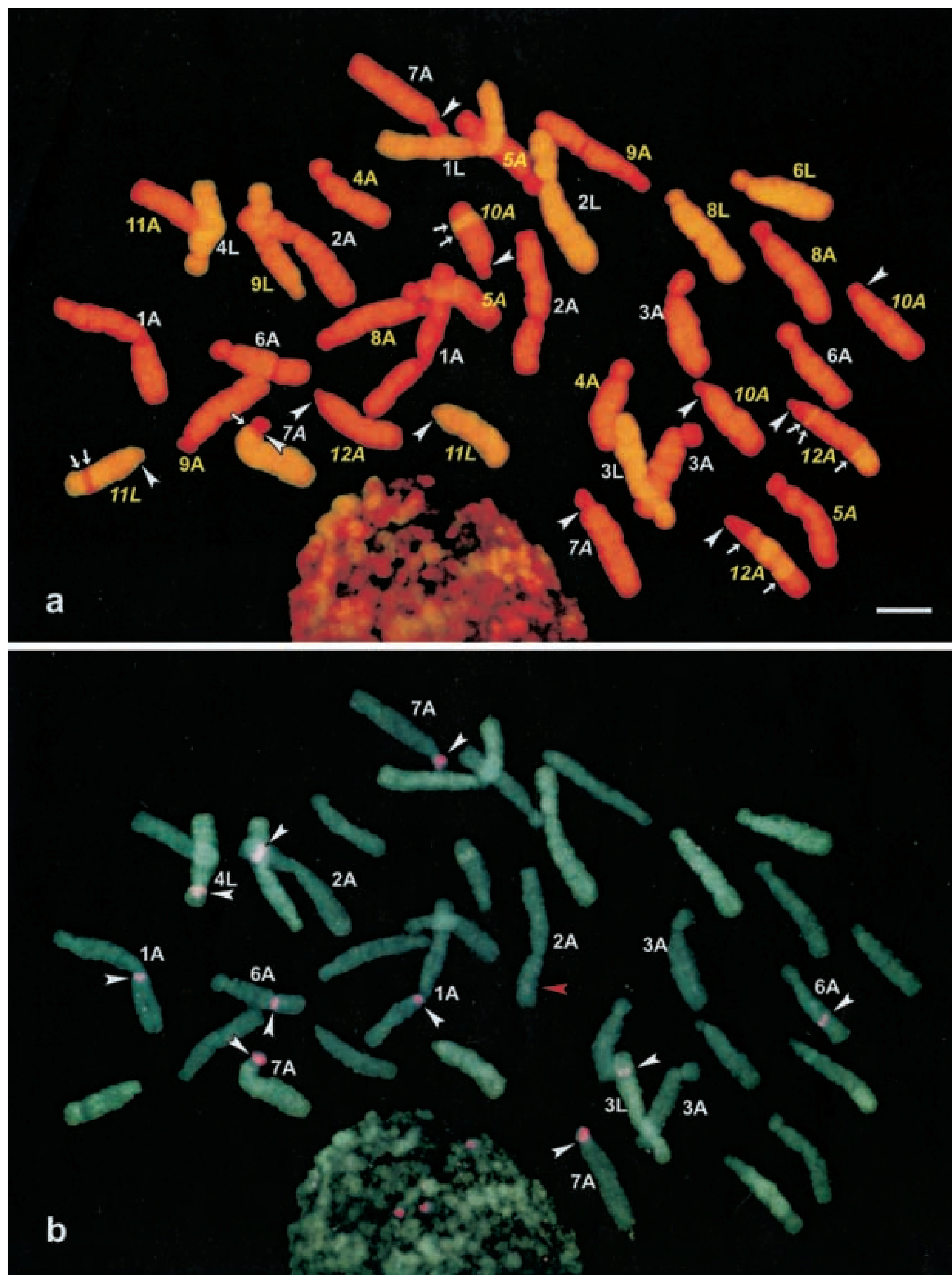

Fig. 4 a Triploid (2n=3x=36) chromosome constitution of $BC_1(ALA_1)$, derived through the functioning of a 2n-gamete that originated through IMR. There were 27 centromeres of the A-genome and 9 centromeres of the L-genome, both in odd numbers. Five recombinant chromosomes with ten breakpoints are indicated. *Arrows* mark the recombination breakpoints. *White letters* indicate a confirmed chromosome number based on pTa71 (45 S rDNA sequence) detection (as in Fig. 2b), and *yellow letters* represent an assumed chromosome number based on observation of the length of the short arm. *Letters* in *italics* represent the substituted chromosome numbers, indicating that all were paired as bivalents during meiosis I. *Arrowheads* indicate the centromere position of the substituted chromosomes. *Bar*, 10 μm. **b** Detection of 45 S rDNA gene sequence (pTa71) on the same chromosome complement as Fig. 2a. *Arrowheads* indicate the position of rDNA hybridisation on the nucleolar-bearing chromosomes. A *red arrowhead* on the short arm of Asiatic chromosome 2 showed a faint signal of rDNA but can not recognisable in this figure

chromosome markers revealed that chromosome 7 of the L genome was substituted for by its homoeologous Asiatic chromosome (arrowheads in Fig. 4b). By measuring the length of the short arms and shape of individual chromosomes, we established that one of the Asiatic chromosomes (no. 11) and four of the *L. longiflorum* chromosomes (nos. 5, 7, 10 and 12) were substituted for with their homoeologous ones (Fig. 5). The five substituted chromosomes showed that Asiatic chromosome 5 had associated with its homoeologue but had no homoeologous recombination, that *L. longiflorum* chromosome 11 and Asiatic chromosomes 7 and 10 had recombinations in one chromosome and that Asiatic chromosome 12 had recombinations in both chromosomes. Because of IMR, in which bivalents (later becoming half-bivalents) always disjoin reductionally as in SDR and univalents divide equationally as in FDR simultaneously in the same PMC, the *L. longiflorum* genome has gained one and lost four chromosomes, resulting in +1–4=–3 chromosomes. In the Asiatic genome, the opposite situation was the case, resulting in +4–1=3 additional chromosomes (Figs. 4a, b, 5).

An interesting feature of all BC_1 individuals was the presence of up to seven recombinant chromosomes per complement showing L/A (or A/L) crossover sites (Table 5). Most of these recombinations occurred at

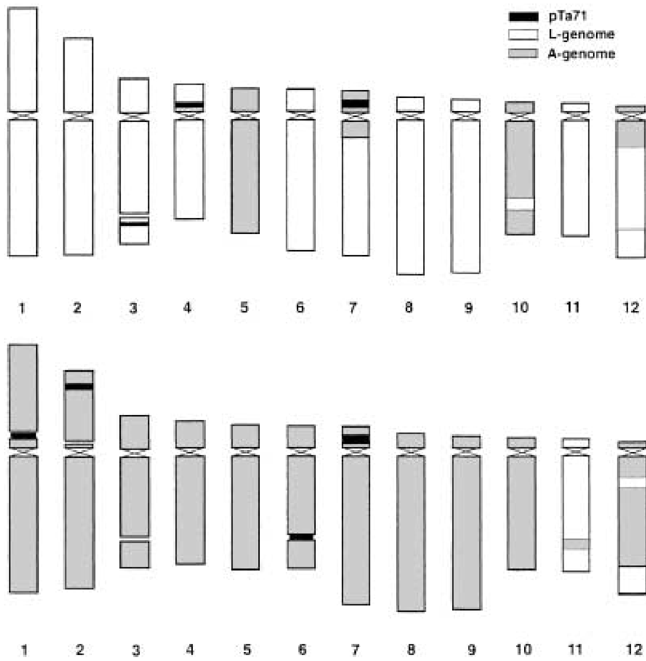

Fig. 5 Ideogram of GISH karyotype of triploid ALA_1, 901122–1(BC_1), represents the chromosome constitution of the IMR 2n-gamete including recombination break-points. Twelve intact Asiatic chromosomes originating from the female are excluded in this figure. The rest of 24 chromosomes are classified into nine L- and 15 A-genome chromosomes originating from the 2n-gametes producing the LA_1 hybrid. An odd number of parental chromosomes resulting from IMR and recombination break-points could be determined in Fig. 4a. The pTa71 probe was used for the detection of 45 S rDNA sites on the NORs bearing chromosomes of both species, which facilitated their identification in Fig. 4b

proximal sites (15 in all) and a few at interstitial and distal regions (Fig 4a). Two types of recombinant chromosomes were distinguished: (1) those with an L-centromere and an A-chromosome segment(s) and (2) the reciprocal type with an A centromere and L-segments, which are indicated as L/A and A/L types, respectively (Table 5). Both types of recombinant chromosomes were found in BC_1 plants.

Discussion

From the cytogenetic point of view, two types of meiotic nuclear restitution leading to 2n-gamete formation are known – FDR and SDR (Fig. 3a, b). Several mechanisms, such as semi-heterotypic division (Rosenberg 1927), mitotisised-meiosis (Stebbins 1932), pseudo-homoeotypic division (Gustafsson 1935), aberrant cytokinesis (Ramanna 1974), parallel spindle (Mok and Peloquin 1975) and fused spindle (Ramanna 1979), that produce FDR 2n-gametes have been identified (Ramanna 1979; Vorsa and Bingham 1979; Peloquin 1983; Parrot and Smith 1984). In addition, mechanisms genetically equivalent to SDR, known as premature cytokinesis 1 and 2 (Mok and Peloquin 1975), have been reported (Veilleux 1985; Britognolle and Thompson 1997). It is evident from those reviews that most studies have been confined to dicotyledonous plants like *Brassica*, *Capsicum*, *Glycine*, *Medicago*, *Solanum* and *Fragaria* (Veilleux 1985) and have been relatively rare in monocots (Parrot and Smith 1984). In this context, the present investigation on *Lilium* hybrids and *Alstroemeria* (Kamstra et al. 1999) are pertinent additions to the subject of 2n-gamete formation in monocotyledonous species. For *Alstroemeria* and *Lilium*, *in situ* hybridisation techniques (GISH and FISH) have been used for the determining the origin of 2n-gametes. In these cases the use of GISH and FISH offered a new perspective for the elucidation of restitution mechanisms, the extent of genetic recombination (both crossing-over and chromosome assortment) and the composition of 2n-gametes.

An important feature of restitutional meiosis in plants with successive cytokinesis in meiosis, as in *Lilium*, is how the events of chromosomal division and cytokinesis occur. The cytological observations made in the present study clearly indicate how meiosis is modified. Instead of chromosome disjunction at anaphase I, all univalents and half-bivalents were aligned on a single equatorial plane. The chromosomes divided simultaneously as a group (Fig. 2a), followed by equational wall formation. This observation explains satisfactorily how FDR gametes can occur. When only univalents are formed in a PMC, FDR gives rise to 2n-gametes without recombinant chromosomes. On the other hand, when both univalents and half-bivalents are present, FDR with recombinant chromosomes is formed. Both of these possibilities are evident from the meiotic observations in this study and corroborate the results of BC plants derived from 2n-pollen of LA hybrids (Table 5).

The occurrence of three types (FDR, SDR and IMR) of 2n-gametes can be explained as follows. In the first type, FDR 2n-gametes, without and with-recombination, were formed. In the case of FDR without recombination, meiocytes at metaphase I form only univalents, which are oriented on the equatorial plane in the PMC. The equational division of the centromeres result in diploid sets of chromatids moving to the two poles at anaphase I, and the complete L and A genomes are reconstituted during the first meiotic division. The anaphase in this situation is a modified form of anaphase I because the centromeres divide before telophase I. During modified telophase I, cell-wall formation gives rise to a dyad with two identical 2n-gametes (figure not shown). In the case of FDR with recombination(s), it is assumed that homoeologous crossovers between L and A-homoeologues occurs. Consequently, the meiocytes display both univalents and bivalents at metaphase I. The bivalents always disjoin prematurely at metaphase I, resulting in half-bivalents, and remain aligned on the equatorial plane together with all the univalents. The resulting FDR gametes now include recombinant chromosomes with the complete genome possessing 12 chromosomes (centromeres) of both the L- and A-genome chromosomes.

In the second (SDR) type, 2n- and normal n-spores are formed as follows: after normal reductional division

(anaphase I), a reductional wall is formed during telophase I. Later the equational wall is formed in only one of the two cells during the second division. This leads to a triad with one 2n- (SDR) and two identical (or nearly identical) n-microspores (Fig. 2e). In this case, SDR 2n-gametes contain an even number of centromeres of the parental genomes, but never an odd number.

In the third (IMR) type, numerically disproportionate numbers of chromosomes occur due to a restitution mechanism which cannot be categorised as either FDR or SDR. In this case, although the 2n-gamete can have the euploid number of 24 chromosomes (from chromosome 1–12 in lily), each set has not contributed the same number. This anomalous situation can be explained as follows: during the first meiotic division all univalent(s) divide equationally whereas bivalents disjoin (reductionally) with the half-bivalents moving to both poles as in normal anaphase I. Following that step, cytokinesis takes place, dividing the two groups of chromatids and half-bivalents. Consequently, the sister-centromeres (chromatids) of each of the half-bivalents are included in the same nucleus of the daughter cell (Fig. 2b). The odd number of centromeres of the parental chromosomes cannot be explained by either FDR or SDR. We have coined the term "Indeterminate Meiotic Restitution" (IMR) for this type of anomalous meiotic restitution.

The equational division of univalents in the first meiotic division has often been recorded in plants such as *Taraxacum* (Gustafsson 1935), *Lilium* (Richardson 1936; Ribbands 1937; Asano 1982, 1984), *Chondrilla* (Bergman 1950) and *Solanum* (Ramanna 1983; Jongedijk et al. 1991). Interestingly, the chromosome behaviour of univalents and bivalents in an interspecific hybrid of *Lilium auratum platyphylum*×*L. henryi* observed by Asano (1984) was similar to that observed in the present LA hybrids (Fig. 3c). However, he did not determine the consequences of this division with respect to the type of restitution that could occur. Asano (1984), however, did record a high frequency of restitution and 2n-pollen. In that case, only a low proportion of the 2n-pollen was viable as determined through pollen germination tests (Asano 1984).

Based on traditional cytological and genetical approaches, only two types of meiotic restitution mechanisms FDR and SDR (Fig. 3a, b) – have been recognised until now (Mok and Peloquin 1975; Ramanna 1979; Veilleux et al. 1982). On the basis of the present approach using GISH, however, a new restitution mechanism, "IMR", has been discovered. It has proven that one of the BC_1 plants showed an odd number of parental chromosomes. In this plant, it is evident that genetic recombination can occur during the origin of 2n-gametes, not only through homoeologous crossing-over but also through the assortment of chromosomes (univalents and half-bivalents). This means that wide interspecific hybrids showing disturbed chromosome association such as the one described in the present study have the potential to produce 2n-gametes from FDR, SDR and IMR. Although half-tetrad analysis using traditional genetic markers or molecular markers is helpful for establishing the restitution mechanism and the extent of crossing-over, the cytological approach of the present study is more direct (Fig. 2b).

The frequency of dyad formation (25%, Table 3), stainable pollen (4%, Table 4) and pollen germination (<1%, Table 4) in the LA_1 hybrid showed a dramatic decrease compared to that found in LA_2 and LA_3. This is probably due to an unbalanced chromosome composition through IMR in which homoeologous chromosome replacement is not compensated for. Despite the potential occurrence of various types of 2n-gametes in the diploid hybrids, only a few types seem to be recoverable in the BC_1 progenies. In the case of *Lilium*, both types of FDR gametes (i.e. with and without recombinant chromosomes) (Karlov et al. 1999) and one IMR gamete have been discovered in BC_1 progeny (Fig. 4). However, the frequency with which they were discovered in the progenies may not be proportional to the frequency with which they are produced in the LA hybrids. This might be caused by gametic, zygotic or post-zygotic selections.

For the purpose of introgression, recombination following homoeologous cross-over is the most important event in BC plants (Kamstra et al. 1999; Lim et al. 2000). In a previous study a fertile amphidiploid F_1 hybrid (LLRR) derived from chromosome doubling of the F_1 hybrid of *L. longiflorum*×*L. rubellum* (LR) and its BC_1 and BC_2 progenies were analysed. No intergenomic recombinations between the L- and R-chromosomes were observed in the BC_1 and BC_2 progenies (Lim et al. 2000). Using such an approach, the introgression of whole individual chromosomes rather than recombinant segments is obtained after further backcrossing. Thus, mitotic polyploidisation in these hybrids does not seem to be the ideal strategy for the introgression of specific desirable characters by recombination of the alien chromosome segments. However, in the present LA hybrids with the natural potential of producing 2n-gametes, considerable numbers of homoeologous recombinations were achieved. Chromosome analysis of the eight BC_1 plants, Asiatic hybrids×(*L. longiflorum*×Asiatic hybrid), revealed 34 homoeologous recombination break-points, ranging from zero to ten cross-overs per individual BC_1 plant (Table 5). Under these circumstances, at the diploid level, the homoeologous chromosomes are virtually "forced" to pair and to produce cross-overs, resulting in intergenomic recombination.

Eight BC_1 plants were investigated by GISH analysis. The chromosome constitution of all eight BC_1 plants ($ALA_{1\sim8}$ in Table 5) clearly showed that the LA_1, LA_2 and LA_3 hybrids in all cases had contributed 2n-gametes to the progeny which contained an euploid chromosome number, i.e. 24. All of the resulting BC_1 plants were triploid (2n=3x=36) and tetraploid (2n=4x=48), originating from both abnormal micro- and macrosporogenesis leading to 2n-gametes. All plants were the results of FDR 2n-gametes, and only one of them originated from an IMR 2n-gamete. This means that either the IMR 2n-gametes are less viable or that their transmission rate is

lower than that of the FDR gametes. The hypothesis of IMR does not seen to be unique to the LA hybrid. Recently, D'Hont et al. (2000) reported the genome constitution of some cultivars of banana based on GISH results. In one clone, Pelipita (2n=3x=33), they found eight of A-genome and 25 B-genome chromosomes instead of the expected 11A and 22B. Although the mode of origin of this cultivar is not mentioned in the article, it is interesting to note that a euploid (3×) chromosome has originated despite the unequal or unbalanced composition of the constituent genomes. Indeed, this will be the expected consequence of IMR.

Another aspect, which needs to be mentioned, is the usefulness of 2n-gametes in breeding. Most of the work so far has been focused on the use of 2n-gametes for breeding autopolyploids or the polysomic polyploids of alfalfa, potato, clover and many other crops (Bingham and McCoy 1979; Peloquin 1982; Mariani and Tavoletti 1992a, b). In these cases, the value of FDR gametes is in transferring heterosis and parental gene combinations intact in sexual polyploids. However, little attention has so far been paid to the systematic use of 2n-gametes in the breeding of allopolyploids or disomic polyploids. Even in the absence of systematic breeding efforts there have been numerous instances in horticultural crops where sexual polyploids from distant interspecific hybrids have given rise to valuable cultivars (Ramanna 1992; Van Tuyl 1997). In these cases, 2n-gametes have been most useful for combining genomes of distant taxa in spite of the diploid hybrids being "sterile" in the sense of not producing normal n-gametes. Thus, in those cases where the hybrids would have been completely sterile, 2n-gamete formation would be an alternative for utilising such hybrids in breeding. Besides these advantages, the occurrence of intergenomic recombination in the 2n-gametes of the present LA hybrids as well as in *Alstroemeria* (Kamstra et al. 1999) open the possibilities for transferring desirable chromosome segments more purposefully in breeding.

References

Asano Y (1982) Chromosome association and pollen fertility in some interspecific hybrids of *Lilium*. Euphytica 31:121–128

Asano Y (1984) Fertility of a hybrid between distantly related species in *Lilium.* Cytologia 49:447–456

Bastiaanssen HJM, Ramanna MS, Sawor Z, Mincione A, Steen Avd, Jacobsen E (1999) Pollen markers for gene-centromere mapping in diploid potato. Theor Appl Genet 93:1040–1047

Bergman B (1950) Meiosis in two different clones of the apomictic *Chondrilla juncea*. Hereditas 36:297–319

Bingham ET, McCoy TJ (1979) Cultivated alfalfa at the diploid level: origin, reproductive stability and yield of seed and forage. Crop Sci 19:97–101

Britognolle F, Thompson JD (1997) Tansley review no.78. Gametes with somatic chromosome number: mechanisms of their formation and role in the evolution of autoploid plant. New Phytol 129:1–22

D'Hont A, Paget-Goy A, Escoute J, Carreel F (2000) The interspecific genome structure of cultivated banana, *Musa* spp. revealed by genomic DNA *in situ* hybridization. Theor Appl Genet 100:177–183

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

Gustafsson Å (1935) Studies on the mechanisms of parthenogenesis. Hereditas 21:1–112

Harlan JR, De Wet JMJ (1975) On Ö. Winge and a prayer: the origins of polyploidy. Bot Rev 41:361–390

Jongedijk E, Ramanna MS, Sawor Z, Hermsen JG (1991) Formation of first division restitution (FDR) 2n-megaspores through pseudohomotypic division in ds-1 (desynapsis) mutants of diploid potato: routine production of tetraploid progeny from 2x FDR×2x FDR crosses. Theor Appl Genet 82:645–656

Kamstra SA, Ramanna MS, De Jeu MJ, Kuipers AGJ, Jacobsen E (1999) Homoeologous chromosome pairing in the distant hybrid *Alstroemeria aurea*×*A. inodora* and the genome composition of its backcross derivatives determined by fluorescent *in situ* hybridization with species-specific probes. Heredity 82: 69–78

Karlov GI, Khrustaleva LI, Lim KB, Van Tuyl JM (1999) Homoeologous recombination in 2n-gamete producing interspecific hybrids of *Lilium* (Liliaceae) studied by genomic *in situ* hybridization (GISH). Genome 42:681–686

Leitch IJ, Bennett MD (1997) Polyploidy in angiosperms. Trends Plant Sci 2:470–476

Lim KB, Chung JD, Van Kronenburg BCE, Ramanna MS, De Jong JH, Van Tuyl JM (2000) Introgression of *Lilium rubellum* Baker chromosomes into *L. longiflorum* Thunb.: a genome painting study of the F_1 hybrid, BC_1 and BC_2 progenies. Chromos Res 8:119–125

Mariani A, Tavoletti S (1992a) Cytology of 2n gamete formation in the *Medicago sativa* complex. In: Veronesi F, Bullitta S, Caredda S (eds) Ploidy and chromosome manipulation in forage breeding. Proc 17th Meeting Fodder Crops Sect of EUCARPIA, Wageningen Eucarpia pp 60–67

Mariani A, Tavoletti S (1992b) Alfalfa evolution and breeding through 2n gametes. In: Veronesi F, Mariani A, Tavoletti S (eds) Gametes with somatic chromosome number in the evolution and breeding of polyploid polysomic species: achievements and perspectives. Proc Workshop. Assisi, Tipolitografia forziuncola, Italy pp 73–81

Masterson J (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science 264:421–424

Mok DWS, Peloquin SJ (1975) Three mechanisms of 2n pollen formation in diploid potatoes. Can J Genet Cytol 17:217–225

Parrott WA, Smith RR (1984) Production of 2n pollen in red clover. Crop Sci 24:469–472

Peloquin SJ (1982) Meiotic mutants in potato breeding. Stadler Genet Symp 14:1–11

Peloquin SJ (1983) Genetic engineering with meiotic mutants, In: Mulcahy D, Ottaviano E (eds) Pollen: biology and implications for plant breeding. Elsevier, New York, pp 311–316

Ramanna MS (1974) The origin of unreduced microspores due to aberrant cytokinesis in the meiocytes of potato and its genetic significance. Euphytica 23:20–30

Ramanna MS (1979) A re-examination of the mechanisms of 2n gametes formation in potato and its implications for breeding. Euphytica 28:537–561

Ramanna MS (1983) First division restitution gametes through fertile desynaptic mutants of potato. Euphytica 32:337–350

Ramanna MS (1992) The role of sexual polyploidization in the origins of horticultural crops: *Alstroemeria* as an example. In: Mariani A, Tavoletti S (eds) Gametes with somatic chromosome number in the evolution and breeding of polyploid polysomic species: achievements and perspectives. Proc Workshop. Assisi, Tipolitografia forziuncola, Italy pp 83–89

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29:467–501

Ribbands CR (1937) The consequences of structural hybridity at meiosis in *Lilium*×*testaceum*. J Genet 35:1–24
Richardson MM (1936) Structural hybridity in *Lilium martagon* album×*L. hansonii*. J Genet 32:411–450
Rosenberg O (1927) Die Semiheterotypische Teilung und Ihre Bedeutung für die Entstehung verdoppelter Chromozomenzahlen. Hereditas 8:305–358
Soltis DE, Soltis PS (1993) Molecular data and the dynamic nature of polyploidy. Crit Rev Plant Sci 12:243–273
Stebbins GL Jr (1932) Cytology of *Antennaria*. II. Parthenogenetic species. Bot Gaz 94:322–344
Van Tuyl JM (1997) Interspecific hybridization of flower bulbs: a review. Acta Hortic 430:465–476
Van Tuyl JM, De Vries JN, Bino RJ, Kwakkenbos AAM (1989) Identification of 2n-pollen producing interspecific hybrids of *Lilium* using flow cytometry. Cytologia 54:737–745
Van Tuyl, JM, Van Diën MP, Van Creij MGM, Van Kleinwee TCM, Franken J, Bino RJ (1991) Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci 74:115–126
Van Tuyl JM, Meijer H, Van Diën MP (1992) The use of oryzalin as an alternative for colchicine in in–vitro chromosome doubling of *Lilium* and *Nerine*. Acta Hortic 325:625–630
Veilleux R (1985) Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breed Rev 3:253–288
Veilleux RE, McHale NA, Lauer FI (1982) Unreduced gametes in diploid Solanum: frequency and types of spindle abnormalities. Can J Genet Cytol 24:301–314
Vorsa M, Bingham ET (1979) Cytology of 2n pollen formation in diploid alfalfa, *Medicago sativa*. Can J Genet Cytol 21:525–530