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Cytogenetical studies in the bridge cross *Allium cepa* × (*A. fistulosum* × *A. roylei*)

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Abstract *Allium fistulosum* harbours a number of desirable agronomical traits for the breeding of onions. However exploitation of *A. fistulosum* for onion breeding via direct sexual hybridization is problematic. Therefore, we examined if a bridge cross, using *A. roylei* as a bridging species, might provide an alternative. By means of genomic in situ hybridization (GISH) we showed that each of the three parental genomes can be distinguished from the others in interspecific hybrids, suggesting that these genomes contain sufficiently different repetitive DNA families. We succeeded in carrying out multi-colour GISH to metaphase spreads of a first-generation bridge-cross individual [*A. cepa* × (*A. fistulosum* × *A. roylei*)], which is composed of three parental genomes. Recombination between the genomes of *A. fistulosum* and *A. roylei* took place to a large extent: 7 recombinant chromosomes were observed, and it could be shown that the proximal regions of the recombinant *A. fistulosum*/*A. roylei* chromosomes belonged to the former, whereas the distal parts belonged to the latter. The high percentage of bound bivalent arms in metaphase I of pollen mother cells of a fertile bridge-cross individual suggests the introgression of *A. fistulosum* genes, mediated by *A. roylei*, into the genome of *A. cepa*. However, the presence of univalents reflects decreased pairing and recombination between the three genomes. Pollen fertility and pollen-tube growth of the first-generation bridge-cross individual seem to be sufficient to produce a second-

generation bridge-cross (*A. cepa* × first-generation bridge cross) progeny.

Key words Onion · Introgression · Multi-colour genomic in situ hybridization (GISH) · Interspecific crosses · Wild species

Introduction

In the Japanese bunching or Welsh onion (*Allium fistulosum* L.) a considerable number of agronomical traits have been identified which are very beneficial for the breeding of new onion (*A. cepa* L.) cultivars. Resistance to diseases like onion leaf blight (*Botrytis squamosa*; Currah and Maude 1984), pink root (*Pyrenochaeta terrestris*; Netzer et al. 1985) and anthracnose (*Colletotrichum gloeosporioides*; Galvan et al. 1997) are present in *A. fistulosum*. Resistance to an economically important pest in onions, the onion fly (*Delia antiqua*; de Ponti and Inggamer 1984), has also been reported. In addition, *A. fistulosum* has a higher dry matter content, is more pungent and winterhardy, flowers earlier, has a shorter flowering period and has a higher attractiveness for pollinators than onion (van der Meer and van Bennekom 1978). Therefore, ample attention has been given to the successful introgression of genes from *A. fistulosum* to *A. cepa*.

As early as 1931 the first interspecific hybrid between *A. cepa* and *A. fistulosum* was created. It proved to be largely sterile, although occasionally seed set occurred upon selfing (Emsweller and Jones 1935 a,b). Electron microscopical analysis of the synaptonemal complexes of the interspecific hybrid showed that heteromorphic bivalents are present, that the male chiasmata frequency is reduced compared to both parents and that the chiasmata are predominantly interstitial and distal (Albini and Jones 1990). Albini and Jones (1990) also found that synapsis in the centromeric region of the interspecific hybrid is disturbed.

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The backcross (BC₁) of the interspecific hybrid with *A. cepa* usually resulted in a limited number of progeny. This may be due to a pre-fertilization barrier as the growth of the 'cepa' pollen tubes is heavily disturbed in the style of the interspecific hybrid (van der Valk et al. 1991a). Cytogenetical analysis of the backcross showed that at least three paracentric inversions and one translocation are present (Peffley and Mangus 1990, Ulloa-G et al. 1994, 1995). Isozyme and restriction fragment length polymorphism (RFLP) analysis of the backcross revealed homoeologous recombination and distorted segregation (Cryder et al. 1991; van der Valk et al. 1991b; Bark et al. 1994). Analysis of the BC₂ showed that the majority of the plants resembled *A. cepa*, however the reproductive organs of the *A. cepa* type plants were morphologically abnormal; seed set of the BC₂ plants was negligible (Ulloa-G et al. 1995). This led Ulloa-G and co-workers to conclude that nucleo-cytoplasmic incompatibility might be the cause underlying the species barrier between *A. cepa* and *A. fistulosum*.

Bridge crosses might provide a way to circumvent the aforementioned problems (Gwynn et al. 1986; McCoy and Echt 1993; Nanda Kumar and Shivanna 1993). In the case of transferring genes from *A. fistulosum* to *A. cepa*, *A. roylei* is possibly a good candidate to function as a bridge between the other two species (de Vries et al. 1992a). Firstly, *A. roylei* crosses readily with both *A. cepa* (van der Meer and de Vries 1990) and *A. fistulosum* (McCollum 1982). Secondly, *A. roylei* has an intermediate amount of DNA (28.5 pg DNA/2C) as compared to *A. cepa* (33.5 pg DNA/2C) and *A. fistulosum* (22.5 pg DNA/2C) (Labani and Elkington 1987).

In this paper we present a cytogenetical analysis of the first-generation bridge-cross *A. cepa* × (*A. fistulosum* × *A. roylei*). The analysis comprises fluorescence in situ hybridization to mitotic metaphases of interspecific hybrids and the first-generation bridge cross using genomic DNA. A multi-colour genomic in situ hybridization (GISH), used to visualize the three genomes in the bridge cross, is quite unique and has been used previously only in wheat (Mukai et al. 1993). In addition, monochromatic and differential staining of metaphase I of the meiosis of the bridge cross was

performed. The staining methods used are standard techniques, while in situ hybridization techniques for *Allium* have only recently been developed (Ricroch et al. 1992; Hizume 1994; Keller et al. 1995). However, the simultaneous detection of three *Allium* genomes has not been published before. Therefore, this paper also presents a protocol for multi-colour GISH for *Allium*.

Materials and methods

Plant material

Table 1 gives an overview of the species used in our study and the origin of the populations derived from these. *A. cepa* cv 'Maxima' and cv 'Jumbo' were kindly provided by the Dutch breeding companies Van der Have, Rilland, and S&G Seeds, Enkhuizen, respectively. 'Maxima' is a cytoplasmic male-sterile (CMS)-based F₁ hybrid, and 'Jumbo' is an open-pollinated variety. *A. fistulosum* was obtained from the botanical garden of Odessa (Ukraine), and *A. roylei* was a gift of Dr. G. D. McCollum (Beltsville, USA). The interspecific hybrids *A. cepa* × *A. fistulosum* (CF), *A. cepa* × *A. roylei* (CR) and *A. fistulosum* × *A. roylei* (FR) were obtained by hand pollination with a paint brush after emasculation. The bridge-cross population (CC × FR) has been produced by using *A. cepa* cv 'Maxima' as the female parent and the interspecific hybrid (*A. fistulosum* × *A. roylei*) as a male parent. Blow flies were used for pollination to produce the bridge cross. Plants were grown in pots in a frost-free greenhouse.

Genomic in situ hybridization (GISH)

Total genomic DNA was isolated from 2 g of young leaves as described by Rogers and Bendich (1988). Genomic DNA to be used as a probe was sonicated to fragments ranging in size from 0.5 to 10 kb and labeled with either Fluorogreen [FITC (fluorescein isothiocyanate)-11-d-UTP, Amersham] or Biotin-16-dUTP (Boehringer Mannheim) by nick translation. Blocking DNA was obtained by autoclaving genomic DNA for 8 min, which yielded fragments of approximately 100–200 bp. In the hybridization mixture the probe: block ratio varied between 1:50 and 1:60. Somatic metaphase chromosome spreads were made from actively growing root meristems by squashing. Young root tips were pretreated overnight with an aqueous solution of 2 mM 8-hydroxyquinoline at 4°C, fixed in 3:1 (v/v) ethanol-acetic acid for at least 24 h at –20°C, rinsed four to five times in distilled water and finally incubated in 10 mM citrate buffer (pH 4.3) containing 0.1% (w/v) cellulase RS, 0.1% (w/v) pectolyase Y23 and 0.1% (w/v) cytohelicase for 75 min at 37°C. The macerated root tips were spread by dissecting and squashing in a drop of 45% acetic acid.

DNA denaturation and in situ hybridization steps were performed according to Leitch and Heslop-Harrison (1994), Schwarzscher and Heslop-Harrison (1994) and Schwarzscher and Leitch (1994). The hybridization mix contained 50% (v/v) deionized formamide, 10% (w/v) sodium dextran sulfate, 2 × SSC, 0.25% (w/v) SDS, 1 ng/μl probe DNA and 0.05–0.06 μg/μl block DNA. In the case of the bridge cross 1 ng/μl biotin-labeled DNA of *A. fistulosum* and 1 ng/μl FITC-labeled genomic DNA of *A. roylei* was used. The hybridization mix was denatured for 10 min at 70°C and placed on ice for 5 min. After the hybridization mixture was added to the slides a 5-min denaturation step at 85°C was carried out. Hybridization was performed for at least 16 h at 37°C in a humid chamber. Subsequently, the slides were washed in 2 × SSC for 15 min at room temperature, followed by 0.1 × SSC for 30 min at 42°C and 2 × SSC for 15 min at room temperature. Biotin-labeled DNA was detected

Table 1 Origin of the investigated populations

Accession number ^a	Code ^b	<i>n</i> ^c	Origin
91010	CF	13	Jumbo × 84236
86184	FR	121	84236-10 × 79150-2
87271	CR	11	Jumbo × 84037-1
89447	CC × FR	16	Maxima × 86184

^a CPRO-DLO accession number

^b C, F and R represent one genome of *A. cepa*, *A. fistulosum* and *A. roylei* respectively

^c *n*, number of progeny obtained

with CY3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, Pa.) and amplified with biotinylated goat-antistreptavidin (Vector Laboratories, Burlingame, Calif.). FITC-labeled DNA was detected with anti-FITC raised in mouse (Boehringer, Mannheim, Germany) and amplified with anti-mouse FITC raised in horse (Vector Laboratories, Burlingame, Calif.). In the case

of double FISH the chromosomes were counterstained with 1 µg/ml DAPI (4,6-diamino-2-phenylindole). When only FITC-labeled DNA was probed, the chromosomes were counterstained with propidium iodide (1 µg/ml). A triple filter (Zeiss, Munich, Germany) was used for the simultaneous visualization of the three parental genomes.

Fig. 1 Genomic in situ hybridization in three *Allium* interspecific hybrids. **a** *A. cepa* × *A. fistulosum*. Yellow fluorescence: *A. fistulosum* chromosomes, red fluorescence *A. cepa* chromosomes. The NOR-bearing chromosome of *A. fistulosum* is indicated by an arrow. **b** *A. fistulosum* × *A. roylei*. The *A. fistulosum* chromosomes show yellow fluorescence, the *A. roylei* chromosomes red fluorescence. Arrow The NOR-bearing chromosome of *A. fistulosum*. **c** *A. cepa* × *A. roylei*. The *A. roylei* chromosomes show yellow fluorescence, the *A. cepa* chromosomes red fluorescence. **d** Interphase nucleus (arrow) from the interspecific hybrid between *A. fistulosum* and *A. roylei* showing several chromosome areas which originate from the two different parental genomes

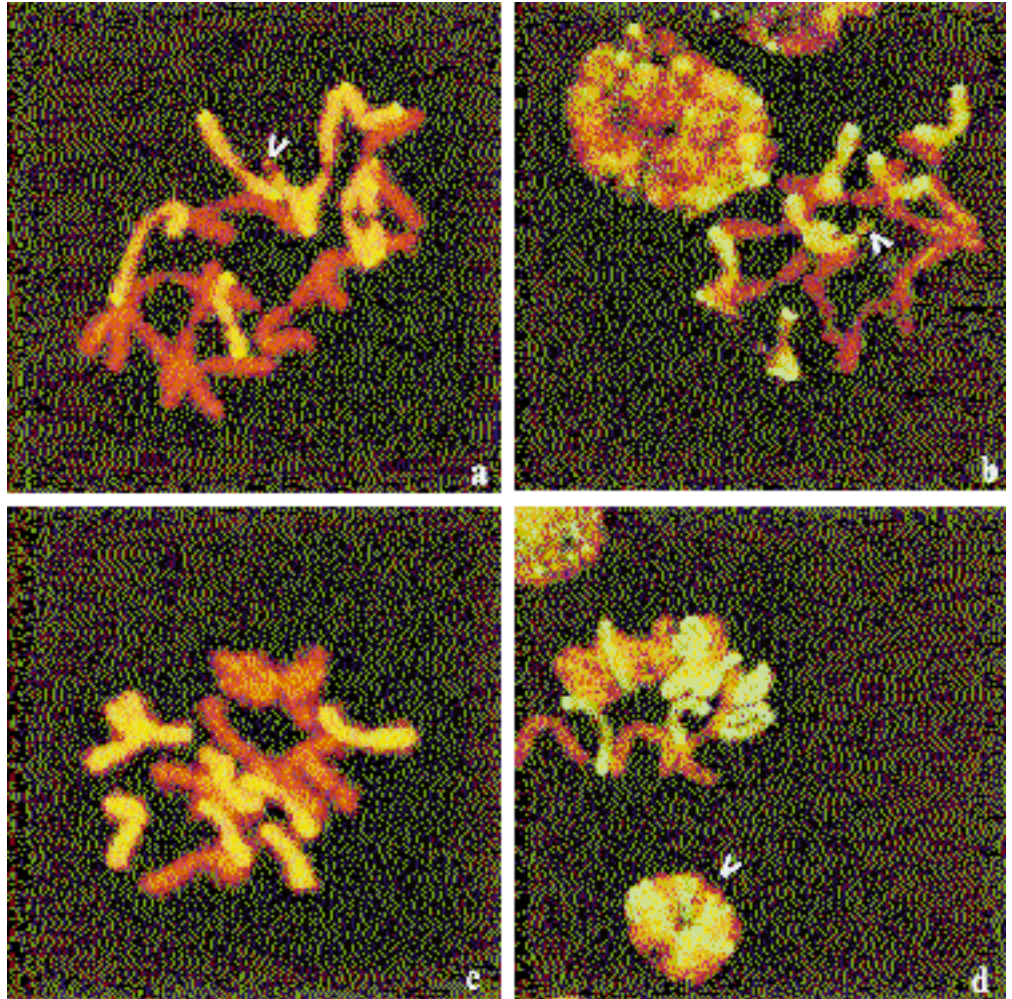
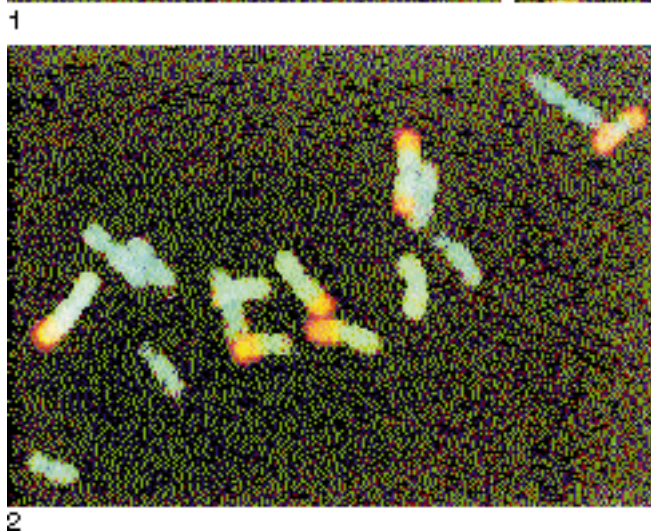


Fig. 2 Simultaneous in situ hybridization of *A. fistulosum* DNA labeled with biotin and enhanced with streptavidin CY3 and *A. roylei* DNA labeled with FITC, using *A. cepa* DNA as a block, in the bridge-cross *A. cepa* × (*A. fistulosum* × *A. roylei*). The eight DAPI-counterstained chromosomes with blue fluorescence belong to *A. cepa*, and one metacentric chromosome with uniform green fluorescence along the entire chromosome belongs to *A. roylei*. Red fluorescence indicates areas hybridized with *A. fistulosum* genomic DNA. On two recombinant RF chromosomes recombination took place at two sites; on the other five chromosomes recombination was observed at a single site



Microsporogenesis

Anthers containing pollen mother cells (PMCs) at metaphase I were fixed in 3:1 (v/v) ethanol-glacial acetic acid at 4°C for at least 16 h. Fixed anthers were rinsed in distilled water, incubated in 4% (w/v) Fe(NH₄)₃-alum for 20 min at room temperature and rinsed in running water for 1 h. The cells were spread by tapping on the cover glass and carefully squashed in 1% Fe-acetocarmine after gently heating the slide. C-banding was performed according to Pijnacker et al. (1992).

Pollen fertility and pollen-tube formation

Pollen morphology and viability were studied in malachite green-acid fuchsin-orange G-stained preparations of microspores (Alexander 1969). Pollen-tube formation was analysed according to Brewbaker and Kwack (1963).

Results

Mitosis

A prerequisite for GISH is that the parental genomes are well-differentiated from each other in order to avoid cross-hybridization. Therefore, we first analysed whether the three parental *Allium* genomes could be distinguished from each other in interspecific hybrids before we analysed the genome organization in the bridge cross.

Interspecific hybrids

One individual from each interspecific hybrid was used for the GISH study. Figure 1a–c shows that all three parental *Allium* genomes can be distinguished from each other in the interspecific hybrids. Consequently, cross-hybridization takes place rarely. Pronounced fluorescence signals are present in the telomeric region of all *A. fistulosum* chromosomes, except for the chromosome arm on which the nucleolar organizer region (NOR) and the satellite are present (Fig. 1a, b). The interphase nuclei of the interspecific hybrid between *A. fistulosum* and *A. roylei* are composed of several chromosomal areas which are derived from the two different genomes (Fig. 1d).

Bridge cross

A multi-colour GISH was performed to analyse the genome organization of the bridge cross. This analysis was carried out using one fertile bridge-cross individual (89447–1). *A. cepa* DNA was used as blocking DNA, while *A. fistulosum* DNA was labeled with biotin (red) and *A. roylei* DNA with FITC (green). The bridge cross displayed 8 blue *A. cepa* chromosomes (DAPI counterstaining), 1 metacentric chromosome with a uniform green fluorescence which belongs to *A. roylei* and 7 recombinant chromosomes (Fig. 2). The 7 recombinant chromosomes can easily be distinguished because they have a green (*A. roylei*) as well as a red (*A. fistulosum*) fluorescence. Two of the recombinant chromosomes contain red fluorescent signals on both arms, while the other 5 chromosomes show red fluorescence only on one arm. Recombination took place in distal and interstitial regions in the interspecific hybrid between *A. fistulosum* and *A. roylei*. Furthermore it was observed that the centromeric region of all recombinant chromosomes originated from *A. roylei*.

Meiosis

Microsporogenesis, pollen fertility and pollen-tube formation was studied using one fertile bridge-cross individual (89447–1).

Microsporogenesis

In the PMCs of 89447–1 only bivalents and univalents but no multivalents were observed. Of the 681 cells scored, 52.8% had eight bivalents, 33.4% had seven bivalents and two univalents and the remaining 13.8% had four or more univalents (Table 2). Some of the bivalents proved to be heteromorphic (Fig. 3). The percentage of bound bivalent arms was 82.6% (Table 2). Most univalents were formed by the smallest chromosomes (Fig. 4).

Table 2 Bivalent configurations in pollen mother cells (PMCs) at metaphase I of a single plant from the bridge cross (*ri* ring bivalent · *ro* rod bivalent · *pu* pair of univalents, *Bba* percentage of bound bivalent arms)

Accession	A ^a	n ^b	Number of PMCs with							Bba ± SE	
			8ri	7ri/ro	6ri/2ro	5ri/3ro	4ri/4ro	1pu	2pu		Other
89447-1	15	681	5	47	86	134	88	228	78	15	82.6 ± 0.9

^a Number of anthers examined

^b n, total number of PMCs examined

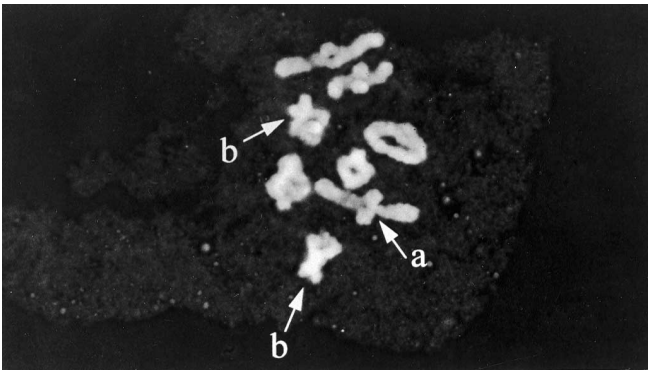


Fig. 3 Acetocarmine-stained metaphase I of the bridge-cross *A. cepa* × (*A. fistulosum* × *A. roylei*). *a* A heteromorphic bivalent, *b* ring bivalents with three chiasmata



Fig. 4 C-banded metaphase I of the bridge-cross *A. cepa* × (*A. fistulosum* × *A. roylei*). The smallest recombinant chromosome has two telomeric C-bands (arrow)

Pollen fertility and pollen-tube formation

Pollen fertility and pollen-tube formation was evaluated in four clones that were obtained by splitting one bridge-cross individual (89447-1). The percentage of fertile pollen varied between 47.2% and 62.9%, and the percentage of pollen which produced a tube varied between 12.5% and 20.6% (Table 3).

Table 3 Pollen fertility and pollen tube growth of different clones of the bridge-cross individual 89447-1

Accession	Clone	Fertility			Tube growth		
		Total	Fertile	%	Total	Growth	%
89447-1	1	580	274	47.2	200	25	12.5
	2	561	256	45.6	287	59	20.6
	3	1060	596	56.2	192	37	19.3
	4	574	361	62.9	417	85	20.4

Discussion

Mitosis

From a phylogenetical point of view *A. cepa*, *A. roylei* and *A. fistulosum* are closely related to each other. All three species belong to the subgenus *Rhiziridium* with *A. cepa* and *A. fistulosum* in section *Cepa* and *A. roylei* in section *Rhiziridium* (van Raamsdonk et al. 1997). However, others have placed the three species in the section *Cepa* (Labani and Elkington 1987). A comparison of cloned repetitive sequences between *A. cepa* and *A. fistulosum* showed a high degree of homology (approximately 80%; Barnes et al. 1985; Irifune et al. 1995). Therefore, in the interspecific hybrids studied here, it was surprising that the three *Allium* genomes could easily be distinguished from each other by means of GISH. In this context Hizume (1994) showed that the genomes of *A. cepa* and *A. fistulosum* could be discriminated by GISH in the interspecific hybrid *A. wakegi*. The results obtained from the study of Hizume and ours therefore point to differences in the repetitive sequences of the three *Allium* species.

We successfully performed a multi-colour GISH in a first-generation bridge-cross individual which is composed of three parental genomes. There are number of differences between the multi-colour GISH technique performed by Mukai et al. (1993) and this study. Our washing procedure was more elaborate, counterstaining (with DAPI) was carried out and a triple filter was used for visualizing all three genomes simultaneously. All in all, the aforementioned modifications led to an improved method for analysing complex triparental genomes.

Analysis of the bridge-cross genome showed that recombination between *A. roylei* and *A. fistulosum* took place to a large extent. This finding is well in line with the results of de Vries et al. (1992b), who observed a high percentage of bound bivalent arms in the interspecific hybrid between both species. The sites of break-points in the recombinant *A. fistulosum/A. roylei* chromosomes proved to be distributed in distal and interstitial regions. This is an interesting observation because it is known that the chiasmata of *A. fistulosum*

are strictly localized adjacent to the centromeres and that the chiasmata of *A. roylei* are randomly distributed (de Vries et al. 1992b). It is possible that the sites of breakpoints in the recombinant chromosomes are determined by a disturbance in synapsis in the centromeric regions, a phenomenon which has also been observed in the interspecific hybrid between *A. cepa* and *A. fistulosum* (Albini and Jones 1990). The observation that the centromeric regions of the recombinant *A. fistulosum/A. roylei* chromosomes originated from *A. roylei* and the distal ends from *A. fistulosum* is remarkable. This might be due to the tendency in interspecific species hybrids to parental genome separation (Bennett 1984; Bennett and Bennett 1992). Parental genome separation in species hybrids is primarily concentric (viz. *Hordeum vulgare* × *Secale africanum*, Finch and Bennett 1981). This type of spatial separation could also have occurred in the interspecific hybrid between *A. fistulosum* and *A. roylei* (Fig. 1d).

Meiosis

The male meiosis of the bridge-cross individual took more or less a regular course. No multivalents were observed, and the percentage of bound bivalent arms (Bba) was considerable (82.6%). The Bba was in the same order of magnitude as the Bba of the interspecific hybrid between *A. cepa* and *A. roylei* (de Vries et al. 1992b). In the latter case it was proven that introgression of *A. roylei* genes to *A. cepa* is possible (Kofeet et al. 1990; Kik et al. 1997). Also in the meiosis of the bridge cross some bivalents proved to be heteromorphic, indicating homeologous pairing during prophase I, which suggests sufficient homology between the three parental genomes to allow chiasma formation. However, univalents were also observed, which means that there is a decreased pairing and recombination among the three genomes.

All in all, the evidence obtained so far suggests that there is a fair chance that introgression of *A. fistulosum* via *A. roylei* to *A. cepa* is possible. This is further strengthened by the fact that pollen fertility and pollen-tube formation is sufficient to allow for a second-generation bridge-cross progeny. In this second-generation bridge cross (*A. cepa* × (*A. cepa* × (*A. fistulosum* × *A. roylei*)) it needs to be proven that introgression of *A. fistulosum* chromosome segments into the *A. cepa* genome is possible. This would be a considerable step forward as compared to the direct sexual hybridization method in exploiting the valuable *A. fistulosum* gene reservoir for the breeding of onion.

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