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Transfer of a male-sterility-inducing cytoplasm from onion to leek (*Allium ampeloprasum*)

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Abstract Two interspecific triploid (AAC) hybrids (84/1-94 and 99/1-94) from crosses between onion [*Allium cepa* (2*n*=2*x*=16, CC)] and leek [*A. ampeloprasum* (2*n*=4*x*=32, AAAA)] were backcrossed to leek in order to transfer a male-sterility-inducing cytoplasm from onion that would enable the production of hybrid leek. GISH evaluations of meiosis in the interspecific hybrids revealed irregularities due to univalent onion chromosomes producing micronuclei from onion chromatin, whereas the pairing of the two sets of leek chromosomes was nearly normal. Attempts to use colchicine to double the chromosome number of the hybrids failed. Backcrosses of 84/1-94 to leek as the pollen parent were not successful. The first backcross of 99/1-94 to tetraploid leek produced 11 BC_1 plants with chromosome numbers between 38 and 41. Identification of parental chromosomes by GISH showed that all eight onion chromosomes and 30–33 leek chromosomes were transmitted to the backcross progenies due to unreduced egg cells. Onion chromosomes were eliminated during the second backcross. Southern hybridization confirmed the transfer of the T-cytoplasm like source of CMS from onion to the BC_2 progenies. After the third backcross to leek, 158 plants were obtained with varying numbers of onion chromosomes and some intergenomic recombinant chromosomes. Alloplasmic leek plants without onion chromatin were selected for further characterization of male sterility and quality traits.

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Introduction

Hybrid cultivars of vegetable crops are preferred for intensive production because of improved earliness, yield potential and homogeneity. Leek (*Allium ampeloprasum* L.) is an important vegetable in Europe (Smilde 1996), and poor uniformity is a major problem (Smith and Crowther 1995). Because of the plant's biennial generation time, tetraploidy (2*n*=4*x*=32), and severe inbreeding depression, the genetic improvement of open-pollinated cultivars is difficult and slow to achieve. Experimental leek hybrids have been shown to have higher yields and a better quality of edible pseudostems (Kampe 1980). The first leek hybrid cultivars were generated using genic male sterility, which requires asexual propagation of the female parent and higher seed costs. Hybrids produced using systems of cytoplasmic male sterility (CMS) would eliminate the need to asexually propagate the female parent of hybrids. However the cytoplasmic diversity of cultivated *A. ampeloprasum* is low, and no source of CMS has yet been identified (Kik et al. 1997; Havey and Lopes Leite 1999).

Among the edible Alliums, onion (*Allium cepa* L.) was the first species for which hybrid cultivars were established using CMS. In onion, two CMS sources are known – the S-cytoplasm (Jones and Clarke 1943) and the T-cytoplasm (Berninger 1965; Schweisguth 1973). T-cytoplasm is closely related to the normal (N) male-fertile cytoplasm of onion (Havey 1995, 2000). The S-cytoplasm was likely transferred to onion from an unknown species through the interspecific hybrid *Allium* × *proliferum* (Moench) Schrad. (Havey 1993, 2000). Alien cytoplasms are known to condition male sterility in the Alliums. The cytoplasm of *Allium galanthum* conditioned male sterility after transfer to onion, shallot (*Allium cepa*, Aggregatum group), and bunching onion (*Allium fistulosum*) (Havey 1999; Yamashita and Tashiro 1999; Yamashita et al. 1999).

A potential method to develop CMS in leek is the transfer of a known male-sterility-inducing cytoplasm from onion to leek. Interspecific sexual (Peterka et al. 1997) and somatic (Buiteveld et al. 1998b) hybrids between S- and T-cytoplasmic onions and leek have been produced. The sexual hybrids were triploids possessing the male-sterile cytoplasm of the female onion parent (Peterka et al. 1997). The fusion hybrids were hexaploids with cytoplasmic mixtures of both onion and leek (Buiteveld et al. 1998a). The next step must eliminate the onion chromosomes from the hybrid to produce plants having the leek nuclear genome combined with the alien male-sterile cytoplasm. The goal of the study reported here was to backcross the sexual onion-leek hybrids to leek and establish the cytoplasm to develop alloplasmic leek.

Material and methods

Hybrid plant material

Two plants, 84/1-94 and 99/1-94, from the seven sexual hybrids between onion and leek obtained by Peterka et al. (1997) were used. The interspecific hybrid 84/1-94 possessed 23 chromosomes and 99/1-94 had the expected number of 24 chromosomes (Fig. 3G). The S-cytoplasm of 84/1-94 and the T-like cytoplasm of 99/1-94 were from the male-sterile onion breeding line ms2 and the Dutch F_1 cultivar 'Summit', respectively. Both triploid hybrids were vegetatively propagated by in vitro formation of multiple shoots, via bulblets in the pseudo-umbel, or plantlets developing at the base of the plants.

Roots of soil-grown triploid hybrid plants were exposed to a colchicine solution at the three- to four-leaf stage of plant development and in vitro plants were grown on colchicine-containing MS (Murashige and Skoog 1962) media. Colchicine concentrations ranged between 0.025% and 0.1%, and the lengths of the treatments varied from 24 h to 96 h.

Flow cytometry

Between 10 and 14 weeks after the colchicine treatments, young leaves from 40 soil-grown plants treated with 0.1% colchicine for 24 h and 13 untreated plants of the hybrid 99/1-94, gynogenetic diploid leek (AA, $2n=2x=16$) (Schum et al. 1993), diploid onion (CC, $2n=2x=16$) and tetraploid leek (AAAA, $2n=4x=32$) were cut with a razor-blade in DAPI (4′, 6-diamidino-2-phenylindole) staining solution (PARTEC) to prepare a suspension of DNA-specific fluorescence-stained nuclei. The nuclear suspensions and a mixed suspension of diploid leek and diploid onion (AA+CC) were filtered through a nylon membrane filter (pore size: 20 μm) and the measurement was done on a linear scale with a PARTEC flow cytometer. G_1 or G_2 peaks of FCM histograms were used to analyze the genome composition per DNA content.

Backcrossing

Shortly before the first flowers opened, the scapes were cut at a length of about 60 cm, transferred to containers containing a commercial nutrient solution and cultured in a growth chamber maintained at 16-h days at 22 °C and 8-h nights at 18 °C. For the first backcross, due to complete male sterility no emasculation was done. Freshly collected leek pollen from different commercial tetraploid cultivars and breeding lines was used to pollinate the umbels of colchicine-treated and untreated hybrid plants. Because of the detection of stainable pollen in some BC_1 plants, flowers were emasculated for the second and third backcrosses. After 4–6 weeks, dark-green ovaries were selected, surface-sterilized, and opened. Black, non-shrunken ovules were cultivated in vitro as described by Peterka et al. (1997).

Mitotic chromosome preparation

Somatic chromosomes were prepared from root-tip cells of in vitro-cultured plants. Excised root tips were treated with 0.15% colchicine at 5 °C for 24 h, fixed in 3:1(v/v) ethanol/acetic acid for 24 h and stored in 70% ethanol. Tips were digested in a solution of 4% cellulase and 1% pectolyase at 37 °C for 7–10 min. Single root tips were transferred into a drop of 45% acetic acid on a slide and gently squashed. The cover slips were removed after the slides were exposed for 20 min to –80 °C. The air-dried slides were used for genomic in situ hybridization (GISH).

Meiotic chromosome preparation

For meiotic analyses, preselected floral buds with anthers containing pollen mother cells (PMCs) at specific stages of meiosis were fixed in 3:1(v/v) ethanol/acetic acid for 24 h at room temperature and stored in 70% ethanol at 4 °C. Anthers were stained with 2% orcein acetic acid for nondifferential staining. For GISH, PMCs were treated in an enzyme mixture (4% cellulase and 1% pectolyase) for 11–15 min at 37 °C. The anther was placed into a drop of 1 μl distilled water to separate PMCs from the rest of the anther tissue. PMCs were squashed in 5 μl 60% acetic acid.

DIG-labelling of genomic onion DNA

For the homogeneous labelling of onion DNA, intermediate products of the amplified fragment length polymorphism (AFLP) procedure (Vos et al. 1995) were used. After digestions of onion DNA with *Eco*RI and *Mse*I, fragments were ligated to *Eco*RI- and *Mse*I-adaptors. Instead of the primers with selective nucleotides normally used in the AFLP process, we used the primers *Eco*RI+0 (5′-GAC TGC GTA CCA ATT C-3′) and *Mse*I+0 (5′-GAT GAG TCC TGA GTA A-3′). The labelling reaction was carried out in a 25-μl aliquot containing 1 × polymerase chain reaction (PCR) buffer, 1.5 m*M* $MgCl_2$, 0.3 μ*M* *Eco*RI+0 primer, 3 μ*M* *Mse*I+0 primer, 200 μ*M* dATP, 200 μ*M* dCTP, 200 μ*M* dGTP, 130 μ*M* dTTP, 70 μ*M* DIG-dUTP, 0.5 μl restriction-ligation solution and 1.25 U *Taq* polymerase (InVitek). Amplification was performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, Calif.) programmed for 2 min 94 °C followed by 20 cycles of 0.5 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and 10 min at 72 °C.

Genomic in situ hybridization

Slides were incubated in RNase (50 μg/ml in 2× SSC) for 40 min at 37 °C. Three 5-min washes in 2× SSC were followed by proteinase K treatment (1 μg/ml in 20 m*M* Tris–HCl, pH 7.4, 2 m*M* $CaCl_2$) for 15 min at 37 °C. For post-fixation, the slides were incubated in 4% paraformaldehyde, 2× SSC for 10 min, then rinsed three times, each for 5 min, in 2× SSC, dehydrated in first 70% and then 96% ethanol, and air-dried. The hybridization mixture (20 μl/slide) contained 10 ng/μl of PCR-DIG-labelled onion DNA, 200 ng/μl of leek competition DNA sheared by autoclaving for 5 min, 500 ng/μl sonicated salmon sperm DNA, 10% (w/v) dextran sulphate, 50% (v/v) deionized formamide, 0.25% SDS, and 2× SSC. The chromosomes and the hybridization mixture were denatured together at 80 °C for 10 min and incubated overnight at 37 °C in a wet chamber. Three post-hybridization washes were carried out in 50% formamide, 1× SSC at 37 °C and three washes in 0.5× SSC, at 50 °C and for 5 min each. DIG-labelled DNA was detected by anti-digoxigenin-FITC from sheep (Roche, Mannheim) for 30 min at 37 °C.

The chromosomes were counterstained with propidium iodide and mounted in vectashield (Vector). Images were captured directly by a CCD camera system (Kappa) on a Nikon Optiphot-2 fluorescence microscope.

Analysis of cytoplasms

To recognize the parental onion and leek cytoplasms as well as to discriminate the two sterile onion cytoplasms, S and T, we utilized polymorphisms in the chloroplast (cp) DNA. The cytoplasms of leek, the onion hybrid Summit, the interspecific hybrid 99/1-94, BC_1 progeny 127/1-96, and 11 BC_2 progenies from crossing 99/1-94 to leek were established using DNA-gel blot hybridizations to reveal polymorphisms in the cpDNA (Havey 1993).

Results

Effects of colchicine treatment

We attempted to produce interspecific hybrid plants with doubled chromosome numbers for backcrossing to leek. The effect of the colchicine treatment was analyzed by flow cytometry of young leaves at approximately 4 weeks before flowering. The DNA content of the onion (C) genome was higher than that of leek (A) genome (Fig. 1A–C), as was expected due to known nuclear DNA amounts of 16.9 (*A. cepa*, 1C value) and 13.2 pg (*A. ampeloprasum*, one half of 1C value), respectively (Bennett and Leitch 1995). The DNA content of the triploid hybrids fell, as expected, almost exactly between that of the diploid onion and tetraploid leek (Fig. 1B, D, E). Among the 40 colchicine-treated hybrid plants, only three plants showed divergent distributions of DNA content as compared to non-treated plants; these three showed a visibly increased triploid G_2 peak (Fig. 1F).

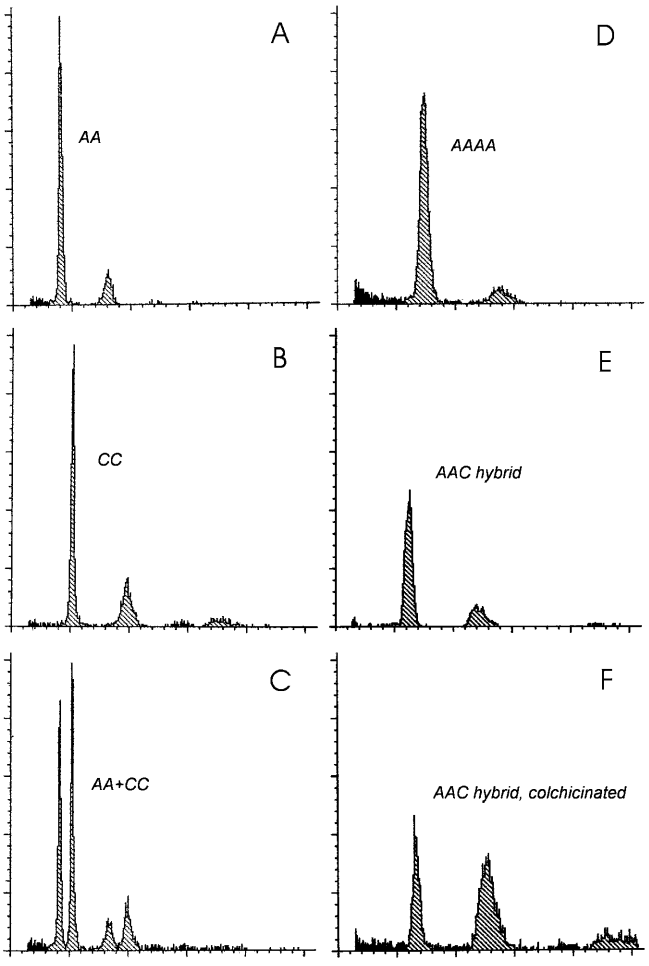


Fig. 1A–F Comparison of DNA contents between parents and interspecific onion-leek hybrid by flow cytometric analysis. **A** Diploid leek (*AA*), **B** diploid onion (*CC*), **C** mixture of both (*AA+CC*), **D** tetraploid leek (*AAAA*), **E** triploid onion-leek hybrid (*AAC*), **F** onion-leek hybrid with modified peaks after treatment with colchicine. *Ordinate* Number of nuclei, *abscissa* DAPI flourescence

Meiosis of interspecific hybrids

The meiotic studies of hybrid 99/1-94 were carried out to compare the ploidy level in the PMCs with the results of the flow cytometric studies using somatic leaf tissue and to study the transmission of onion chromosomes. In PMCs from all of the colchicine-treated and non-treated plants, eight leek bivalents and eight onion univalents consistently appeared at diakinesis (Figs. 2B, 3A, B). This contrasts to the results of the flow cytometric analyses, which indicated some chromosome doubling in vegetative leaf tissue.

The leek bivalents in the hybrids possessed localized chiasmata, as also observed in diploid leek (Fig. 2A). When the leek bivalents moved towards the equatorial plate, the onion univalents arrested at the periphery of the nucleus (Fig. 3C). At late anaphase I, following migration of the leek chromosomes to the poles, four to eight onion chromosomes prematurely separated into chromatids (Figs. 2C, 3D). The retarded movement of onion chromatids compared to the leek chromosomes resulted frequently in their exclusion from the daughter nuclei and to micronuclei formation in the dyad stage (Figs. 2E, F, 3E, F). The number of micronuclei in the dyads ranged from 0 to 17, suggesting that they were not only from onion chromatids but that in some cases also leek chromosomes were involved (Table 1). This meiotic instability of leek chromosomes was detected in the trip-

Table 1 Number of micronuclei in dyads and tetrads of pollen mother cells of two triploid onion-leek hybrids

Hybrid	Number of dyads	Average number of micronuclei/dyad	Range	Number of tetrads	Average number of micronuclei/tetrad	Range
84/1-94	297	2.9	0–11	605	2.5	0–12
99/1-94	504	7.0	0–17	791	5.6	0–15

Table 2 Results of first backcrosses of onion-leek hybrids to leek

Onion-leek hybrid	Year of cross	Umbels pollinated[a]	Ovaries harvested	Ovules prepared	Embryos germinated	Plants developed
99/1-94	1996	55	43	63	8	9[b]
99/1-94	1997	112	5	9	2	2
84/1-94	1997	140	0	0	0	0
Total		307	48	72	10	11

[a] A minimum of 100 flowers per umbel
[b] From one twin embryo two plants developed

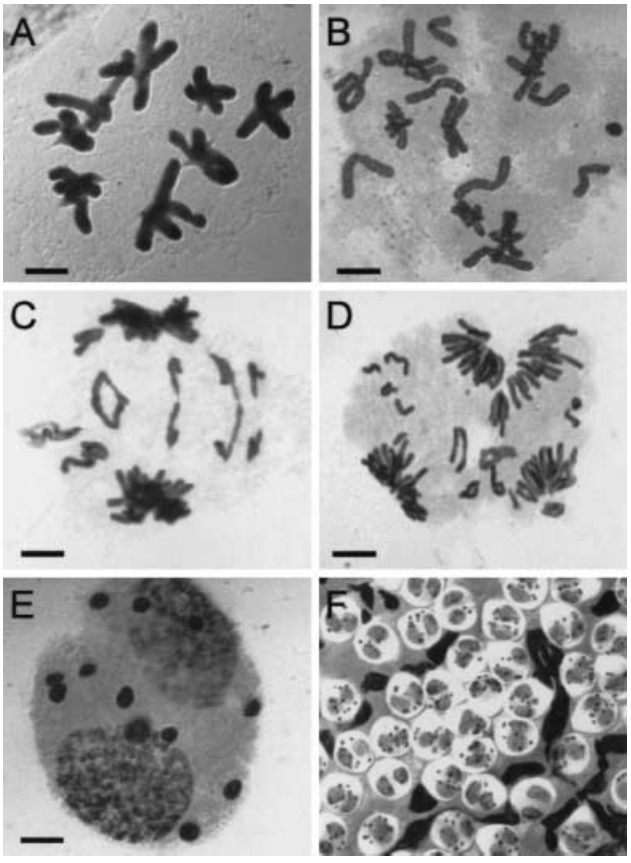


Fig. 2A–F Meiosis in PMCs of diploid leek (**A**) and triploid onion-leek hybrid 99/1-94 (**B–F**). **A** Diakinesis with eight crosslike bivalents, **B** diakinesis with eight crosslike bivalents and eight univalents, **C** delayed premature separation of chromatids at anaphase I, **D** laggards in anaphase II, **E** micronuclei in telophase I, **F** dyads. *Bar*: 10 μm

loid PMCs (Fig. 3E). Laggard chromosomes were observed at a high frequency during anaphase II (Fig. 2D). The average number of micronuclei in the tetrad stage was lower than in the dyads (Table 1). Hybrid 99/1-94 had twice as much micronuclei than 84/1-94. The triploid hybrids produced tetrads, but no functional pollen developed.

First backcross to leek

Forty-eight ovaries were harvested from more than 30,000 pollinated flowers, from which 11 plants were obtained (Table 2). Most of the approximately 300 hybrid plants used for backcrossing had been colchicine-treated. Nevertheless, the first backcross resulted in a very low seed set of 0.04 plants per umbel. Nine progenies in 1996 and two progenies in 1997 were produced from crosses using colchicine-treated and untreated plants of hybrid 99/1-94. Because flow cytometric and meiotic investigations on the hybrid plants showed no evidence of chromosome doubling, colchicine most likely did not enhance the female fertility of hybrid plants. No progenies were obtained from backcrosses of 140 umbels of colchicine-treated hybrid 84/1-94 plants to leek.

It is unlikely that the backcross plants originated from the fertilization of reduced egg cells in the triploid hybrid. The chromosome compositions of all backcross plants were similar, with eight onion chromosomes and four sets of leek chromosomes (Table 3, Fig. 3I). This suggests that all backcross plants were the fertilization products of unreduced egg cells. One BC_1 plant, 93/1-97, had one intergenomic recombinant chromosome.

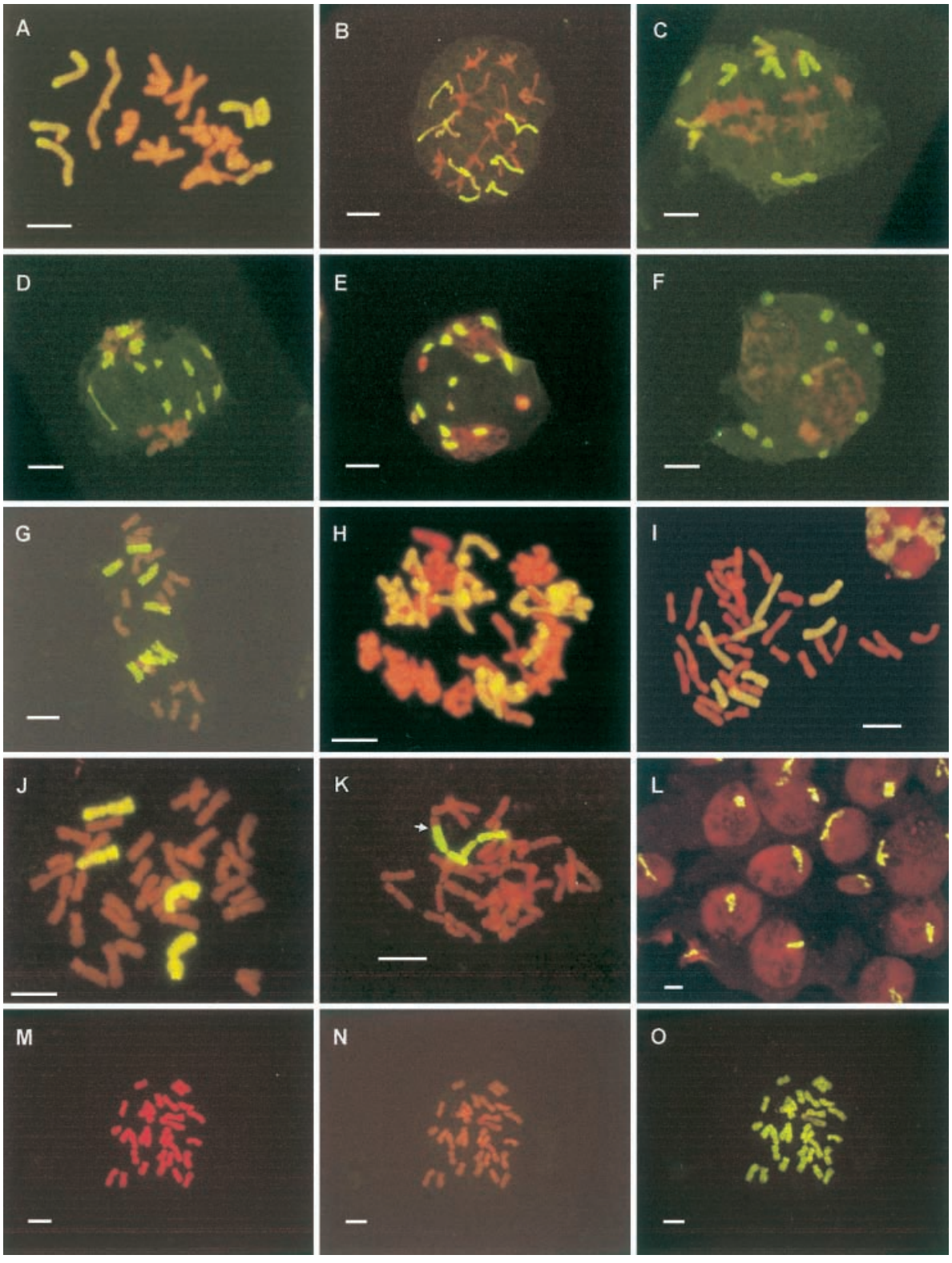
The number of leek chromosomes in BC_1 progenies varied from 30 to 33. Only three of the eleven BC_1 plants had the regular leek chromosome number of 32, indicating a high degree of instability in gamete formation for the tetraploid leek. Recently, a more thorough meiotic study of normal leek varieties revealed unexpectedly high frequencies of multivalents and univalents at metaphase I (Jones et al. 1996), which may be the cause of aneuploid meiotic products.

Second and third backcross

The BC_1 plants were backcrossed to leek in 1998 and again after one cycle of propagation in 1999. From 104 pollinated umbels, 79 BC_2 plants were produced, yielding about 0.8 plants per umbel (Table 4). Although this is low, it is much higher than in the BC_1 generation.

In the BC_2 generation, the numbers of onion chromosomes varied between 0 and 8 (Table 5, Fig. 3J–L). On average, 1.6 onion chromosomes per plant were trans-

Fig. 3A–O GISH of triploid hybrid 99/1-94 (**A–G**), hexaploid PMC of hybrid 84/1-94 (**H**), backcross derivatives of 99/1-94 with onion chromosomes (**I–L**) and an alloplasmic leek (**M–O**). **A** PMC at diakinesis with labelled onion DNA reveals that all eight univalents are from onion (*yellow*) and the eight bivalents from leek (*red*), **B, C** PMCs with undigested cytoplasm showing the peripheric and distant location of onion univalents in triploid hybrid 99/1-94, **D** PMC at late anaphase I showing premature sep- ▶



aration of onion chromatides, **E** anaphase I with several onion (*yellow*) as well as two leek (*red*) laggards, **F** dyad with ten onion micronuclei, **G** mitotic cell with eight onion and 16 leek chromosomes, **H** metaphase I showing regular pairing of onion bivalents in unreduced gametic hybrid cells, **I** mitotic cell of the BC_1 plant 136/1-96 having eight onion and 31 leek chromosomes, **J** BC_2 plant with four onion and 32 leek chromosomes, **K** BC_2 plant with one onion, one intergenomic recombinant (*arrow*) and 32 leek chromosomes, **L** BC_2 plant with one added onion chromosome showing its integrated location within the interphase nuclei, **M–O** fluorescence signals in a mitotic cell of an alloplasmic leek with 32 chromosomes after staining with propidium iodide (**M**), onion genomic DNA probe (no signals) (**N**) and rehybridized with labelled leek genomic DNA (**O**). *Bar*: 10 μm

Table 3 Chromosomal constitution of 11 plants from the first backcross of triploid onion-leek hybrid 99/1-94 to tetraploid leek

Female hybrid plant	Colchicine-treated	BC_1 plant	Total chromo-some number	Number of onion chromo-somes	Number of leek chromo-somes	Inter-genomic recombinant chromosomes
99/1/16C-94	Yes	116/1-96	40	8	32	
		116/2-96	40	8	32	
		116/3a-96[a]	41	8	33	
		116/3b-96[a]	41	8	33	
99/1/51C-94	Yes	121/1-96	40	8	32	
99/1/30C-94	Yes	127/1-96	38	8	30	
99/1/54C-94	Yes	131/1-96	39	8	31	
99/1/17C-94	Yes	133/1-96	41	8	33	
99/1/68-94	No	136/1-96	39	8	31	
99/1/62a-94	No	93/1-97	39	8	30	1
99/1/62b-94	No	214/1-97	39	8	31	

[a] Twins

Table 4 Results of crossing experiments for the second backcross of onion-leek hybrid 99/1-94 to leek

BC_1 plant	Umbels pollinated	Ovaries harvested	Ovules prepared	Embryos germinated	BC_2 plants developed
116/1-96	16	2819	68	14	10
116/2-96	3	298	13	3	3
116/3a-96	3	430	26	9	8
116/3b-96	2	312	30	11	8
121/1-96	3	464	18	10	7
127/1-96	26	2846	98	21	13
131/1-96	5	360	28	7	5
133/1-96	3	227	7	3	2
136/1-96	41	1184	125	31	22
93/1-97	1	30	8	4	0
214/1-97	1	117	8	1	1
Total	104	9087	429	114	79

Table 5 Frequency of plants with varying numbers of transmitted onion chromosomes in the BC_2 progenies of different BC_1 parents

BC_1 parent	Number of BC_2 plants	BC_2 plants analyzed	Plants with onion chromosome number								
			0	1	2	3	4	5	6	7	8
116/1-96	10	9	1	3	3				1[b]		1[b]
116/2-96	3	3	1	1	1[a]						
116/3a-96	8	7	3	2	1	1					
116/3b-96	8	8	4	2	1	1					
121/1-96	7	7	3	3		1[b]					
127/1-96	13	9	5	1	2	1[a]					
131/1-96	5	4	1	1		2					
133/1-96	2	2	1	1							
136/1-96	22	22		3	6	6	3	2	1		1[b]
214/1-97	1										
Total	79	71	19	17	14	12	3	2	2		2

[a] Plant has an additional intergenomic recombination chromosome

[b] Plants with a total chromosome number above 50 are from unreduced gametes

mitted to the BC_2 generation. The distribution of onion chromosomes was similar in all progenies of the BC_1 plants with exception of that of plant 136/1-96 which showed a higher transmission rate. Among the 71 plants analyzed, most possessed zero to three onion chromosomes. Alloplasmic leeks probed with onion and leek genomic DNA did not contain any onion chromatin (Fig. 3M–O).

Four plants had more than 50 chromosomes, with six instead the expected four leek genomes and three to eight onion chromosomes. These plants apparently resulted from unreduced gametes of either the BC_1 plant, if they had a complete onion genome, or of the backcross leek parent, when they had an incomplete set of onion chromosomes. In two plants, intergenomic recombinant chromosomes were detected by GISH (Fig. 3K).

The cytoplasms of Summit, the interspecific hybrid 99/1-94, the BC_1 progeny 127/1-96, and 11 selected BC_2 progenies were identical and showed a clear polymorphism to the leek parent (Fig. 4). The onion population

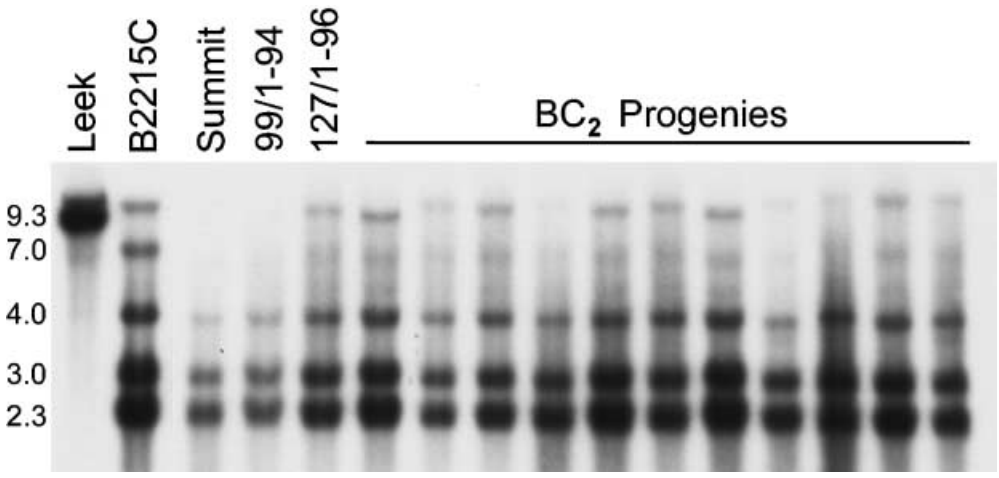


Fig. 4 Polymorphisms in the cpDNA revealed with probes 12b&c hybridized to *Eco*RV digests (Havey 1993) for leek cv. Pollux (*lane 1*), the onion inbred B2215C (*lane 2*), cv. Summit (*lane 3*), the onion-leek hybrid 99/1-94 (*lane 4*), the BC_1 progeny 127/1-96 (*lane 5*) and 11 BC_2 progenies (*lanes 6–16*). The presence of the 3.0- and 4.0-kb fragments indicate that the male-sterile cytoplasm transferred from hybrid onion cv. Summit was not S-cytoplasm. B2215C possesses both normal male-fertile and S-cytoplasms

Table 6 Results of backcrossing BC_2 plants to leek

BC_2 plant no.	Number of chromosomes (leek + onion)	Umbels pollinated	Ovaries harvested	Ovules prepared	Embryos germinated	BC_3 plants developed
14/1-98	32+4	1	145	12	6	6
20/4-98	32+1	1	54	17	12	9
21/1-98	32+2	2	362	66	40	27
21/2-98	31+3	1	106	80	35	26
21/3-98	32+3	1	62	2	2	2
21/4-98	32+6	1	150	2	1	1
Total		7	879	179	96	71

Table 7 Transmission of onion chromosomes from two BC_2 plants to their BC_3 progenies

BC_2 parent	Onion chromosomes	Number of BC_3 plants	Analyzed	Number of plants observed (expected) with onion chromosome number			
				0	1	2	3
21/1-98	2	27	19	16 (4.75)	3 (9.5)	0 (4.75)	
21/2-98	3	26	15	11 (1.875)	4 (5.625)	0 (5.625)	0 (1.875)

B2215C possesses a mixture of N- and S-cytoplasms (Havey 1995) and was used as a control. The hybridization patterns of cpDNA with the probes showed that there are no differences between N- and T-cytoplasm (Havey 1993). The 3.0-kb and 4.0-kb fragments are present in T-cytoplasm and absent in S-cytoplasm, whereas the 7.0-kb fragment is only present in S-cytoplasm. As a result, Summit possesses the T-cytoplasmic-like source of CMS (Havey 2000), and this male-sterile cytoplasm was successfully transferred to leek.

Six BC_2 plants with one to six onion chromosomes were backcrossed again to leek. From the seven umbels pollinated, 71 BC_3 plants were obtained (Table 6). The seed set in the third backcross was more than tenfold higher than in BC_2. The transmission of the onion chromosomes was analyzed among the progenies of two BC_2 plants, which derived from the same BC_1 parent and possessed two and three onion chromosomes, respectively (Table 7). The observed mean transmission of 0.16 onion chromosomes per plant for 21/1-98 and of 0.27 for 21/2-98 is much lower than the random expectations of 1 and 1.5 chromosomes per plant, respectively.

The low transmission rate of univalent onion chromosomes resulted in a high percentage of alloplasmic leek plants at the second and third backcross generations (Tables 5, 7). Altogether, more then 40 alloplasmic leeks were obtained and vegetatively multiplied.

Discussion

Chromosome doubling in the hybrids

Chromosome doubling of interspecific hybrids was believed to be a prerequisite for successful backcrossing. Following in vitro colchicine treatments, we found no evidence of chromosome doubling in the gametophyte, whereas in root tips some chromosome doubling had taken place. In gynogenic onions, root tips and shoot apices were observed to respond differently to colchicine treat-

ments (Campion et al. 1995). One reason for this could be the higher number of root meristems as compared to only one apical meristem or the the more inaccessible location of the shoot apex. In the few plants which had doubled chromosome numbers in leaf tissues, as shown by flow cytometry, no doubling occurred in generative flower parts. These leaves might be ploidy chimeras with chromosome doubling only in cell layers not contributing to sporogenous tissue.

Unreduced hybrid egg cells

The observed failure of colchicination, the occurrence of backcross progenies from non-colchicine-treated hybrid plants and the low frequency of fertilizations indicate that the BC_1 plants resulted from the fertilization of unreduced egg cells. The regular formation of eight onion and 16 leek bivalents in hybrid microgametogenesis was found in doubled PMCs of colchicinated plants of hybrid 84/1-99 (Fig. 3H). Some BC_2 offspring arose from unreduced egg cells of BC_1 plants (Table 5). Diplospory is a known phenomenon in the genus *Allium*. Levan (1933a) found huge pollen grains with doubled chromosome numbers in several *Allium* species. Unreduced egg mother cells were observed in *A. nutans* (Håkansson 1951), *A. odorum* (Håkansson and Levan 1957), and *A. tuberosum* (Gohil and Kaul 1981). In the diplosporous apomict *A. tuberosum*, the percentage of unreduced egg mother cells was as high as 98% (Kojima and Nagato 1992).

Increase of seed set in consecutive backcrosses

The seed set of interspecific hybrids and their derivatives reflects their different female fertilities. The complete female sterility of hybrid 84/1-94 could be due to karyotype abnormalities. In this hybrid, loss of a single leek chromosome, deletions and translocations have been observed (Schrader et al. 2000). Hybrid 99/1-94 had one complete onion and two complete leek chromosomal sets and showed after the first backcross a seed set of 0.04 plants per umbel. The seed set on BC_1 plants having an additional two leek chromosomal sets increased to 0.7 after the second backcross to leek. The subsequent rise in seed set after the third backcross to 10.0 plants per umbel could be due to the decreased number of extra onion chromosomes leading to a better endosperm development (data not shown).

Micronuclei formation and transmission of onion chromosomes

The low transmission rate of univalent onion chromosomes resulted in the production of the alloplasmic BC_2 leek plants. Leek plants possessing a single onion chromosome will be a useful tool for genetic analyses of onion.

The number of transmitted onion chromosomes averaged 1.6 for all BC_2 plants, as compared to the expected maximum number of four. This indicates that, on average, 6.4 onion chromosomes (40%) out of the 16 possible daughter chromosomes remained during female meiosis of BC_1 plants having eight onion univalents. The loss of 9.6 daughter chromosomes could be related to the observed number of micronuclei from the univalent onion genome in the PMCs of the triploid onion-leek hybrid 99/1-94. The average number of micronuclei at the dyad and tetrad stage was 7.0 and 5.6, respectively (Table 1), suggesting that 12.6 onion daughter chromosomes per PMC were eliminated. We conclude that either megasporogenesis in the BC_1 plants shows a slower elimination of onion chromosomes than that in the triploid hybrid or that some of the micronuclei in the triploid hybrid must have been reincluded in further cell divisions. In a similar experiment by Shigyo et al. (1996), an average of 3.4 onion chromosomes were transmitted to the BC_2 of a *A. cepa* × *A. fistulosum* cross. This higher transmission rate could be due to the lower ploidy level of diploid *A. fistulosum* as compared to the tetraploid *A. ampeloprasum* genetic background or to its closer phylogenetic relationship to onion (Havey 1991).

Intergenomic recombination

The presence of intergenomic recombinant chromosomes in the backcrosses indicates that chromosome pairing between the univalent onion and some of the tetravalent leek chromosomes occurred in the early stages of egg cell meiosis. Onion and leek differ with respect to the pairing behavior of their metaphase I chromosomes. In onion, chiasmata are formed mainly in the distal and interstitial region of the chromosome arms, whereas leek shows a highly proximal localization of chiasmata (Levan 1933b; Koul and Gohil 1970; Wajahatullah 1994; Khazanehdari et al. 1995; Jones et al. 1996; Stack and Roelofs 1996). Recombined chromosomes were also observed in *A. cepa* × *A. fistulosum* hybrids (Hou and Peffley 2000) as well as in a bridge cross involving *A. cepa*, *A. fistulosum*, and *A. roylei* (Khrustaleva and Kik 2000), where similar differences exist in chiasma localization and DNA amount of the parental genomes.

Development of a CMS system for leek

Molecular analyses of the cpDNAs revealed that the T-cytoplasm-like source of onion CMS was successfully transferred by backcrossing to leek. Future work will establish the male fertility of alloplasmic leek populations and study the development of the male gametophyte and quality attributes of the alloplasmic leeks. Havey (1999) transferred the cytoplasm of *A. galanthum* to onion and observed no nuclear restoration of male fertility. The cytoplasm of *A. galanthum* has also been transferred to shallot (Yamashita and Tashiro 1999) and bunching onion (Yamashita et al. 1999) to develop new sources of CMS. In bunching onion, nuclear restoration of male fer-

tility for the *A. galanthum* cytoplasm was conditioned by a nuclear genomic region transferred from *A. galanthum* during backcrossing. In onion, there are three loci known to affect male-fertility restoration in plants possessing T-cytoplasm (Schweisguth 1973). The existence of these loci or other male-fertility restoration loci in leek will also be determined.

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