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Recombinant chromosomes of advanced backcross plants between *Allium cepa* L. and *A. fistulosum* L. revealed by in situ hybridization

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Abstract Cytological analysis of (*Allium cepa* L.×*Allium fistulosum* L.)×*A. cepa* L. F_1BC_3 plants revealed most plants were diploid with 16 chromosomes. Karyotypes of these plants showed recombinant chromosomes. Fluorescence and genomic in situ hybridization patterns of interspecific F_1 hybrid and F_1BC_3 plants revealed *A. fistulosum* chromosomes or chromosomal segments. A highly repetitive 376-bp DNA sequence and genomic DNA of *A. fistulosum* revealed similar telomeric hybridization sites when hybridized onto *A. fistulosum* chromosomes. Cytogenetic evidence showed that *A. fistulosum* DNA has recombined into the *A. cepa* genome.

Key words Fluorescence in situ hybridization · Genomic in situ hybridization · Interspecific introgression · Japanese bunching onion · Onion

Introduction

The *Allium cepa* genome is 27% larger than that of *Allium fistulosum* (Jones and Rees 1968), leading to *A. cepa* chromosomes being larger in size than those of *A. fistulosum*. The disparity in chromosome size and the position of the centromeres aids in distinguishing the larger *A. cepa* chromosomes from the smaller *A. fistulosum* chromosomes in mitotic karyotypes, and this allows for meiotic characterization of heteromorphic chromosome pairing. Karyotypes of each species have been established and used for interspecific cytological studies (Peffley et al. 1985; Peffley 1986; Peffley and Currah 1988; Ulloa et al. 1994). Complete homeologous pairing of *A. cepa* and *A. fistulosum* chromosomes is common (Emsweller and Jones 1935; Maeda 1937) but, typical for pairing-control gene heterozygosity, the number of bivalents varies with the parents used in the original cross. In a study of F_1BC_1 plants (Ulloa-G et al. 1994), bivalent frequency ranged from less than half to complete pairing, yet fertility was not restored in any of the plants.

An understanding of genome recombination requires consideration of how DNA is transferred during introgressive hybridization. Tracking the movement of *A. fistulosum* DNA in the early generations of F_1 hybrid-derivatives is difficult as the sterility of the F_1 progeny hinders introgression (Peffley 1986). Tracking of *A. fistulosum* germplasm into *A. cepa* can be done by morphology, but these traits become more ambiguous as backcrossing to *A. cepa* continues (Currah and Ockendon 1988). Moreover, the phenotype does not give any information about DNA movement between species. Molecular markers, such as isozymes (Peffley et al. 1985) and RFLPs (Bark et al. 1994), can differentiate *A. fistulosum* from *A. cepa.* However, these markers still provide limited information regarding genomic recombination on how chromosomes pair and allow for gene flow between the species. Karyotypes give the result of cross-over events as visualized with recombinant chromosomes, but without molecular cytological information it is unclear which segments are contributed from each species.

Fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH) have provided new tools for genomic research in many crops. Alien chromosomes or chromosomal fragments have been identified by GISH with biotin-labeled genomic DNAs in interspecific hybridizations in wheat (Miller et al. 1994; Schwarzacher et al. 1992), barley (Schwarzacher et al. 1989), and cereal crops (Anamthawat-Jonson and Reader 1995). Cytogenetic studies with FISH mapped a *Helianthus* rDNA sequence to the chromosomes of *A. cepa* and *A. fistulosum* (Ricroch et al. 1992), and GISH (Khrustaleva and Kik 1998; Peterka et al. 1997) has also been reported in *Allium*. Recombinant chromosomes in interspecific hybrids of *A. cepa*×*Allium ampeloprasum*

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(Peterka et al. 1997) and *A. cepa*×(*A. fistulosum*×*Allium roylei*) (Khrustaleva and Kik 1998) were evidenced by ISH with biotin-labeled genomic DNA.

In a separate examination (Peffley and Hou 2000) the F_1BC_3 plants investigated in this report were identified as recombinant introgressants possessing morphological and isozyme markers of both *A. cepa* and *A. fistulosum* These recombinant plants are the subject of this report. Biotin-labeled probes of genomic and repetitive DNA hybridizations of these advanced backcross recombinant plants provide a unique opportunity to visualize past DNA recombination events between these two species and document that introgressive hybridization has occurred.

Materials and methods

Plant materials

Sixteen F_1BC_3 plants, generated by backcrossing *A. cepa* to an *A. cepa*×*A. fistulosum* F_1 hybrid (Peffley and Hou 2000), were used for mitotic chromosome counts and karyotypes. Plants of *A. fistulosum* 'Ishikura', *A. cepa* 'New Mexico Sunlite', and their F_1 hybrid '8273' (*A. fistulosum*×A. *cepa*) were used in chromosome preparations for in situ hybridization. F_1BC_3 plants 951027-54 and 951027-97 were also used for this in situ hybridization study.

Morphological observation and isozyme analyses

The 16 F_1BC_3 plants were recorded for morphology as bulbing in *A. cepa* and as non-bulbing in *A. fistulosum*. The fertility of the plants that flowered were recorded in terms of pollen stainability and seed set per umbel. Pollen stainability was tested using microspores following Peffley et al. (1985).

The isozymes investigated were alcohol dehydrogenase (ADH) (E.C.1.1.1.1), phosphoglucoisomerase (PGI) (E.C.5.3.1.9) (both following Peffley et al. 1985), and esterase (EST) (E.C.3.1.1.) (Hou et al. 2000). Plants were scored for the presence of *A. cepa* (c) and *A. fistulosum* (f) alleles. A plant heterozygous for c/c alleles of *A. cepa* and f/f of *A. fistulosum* was scored as c/f.

Mitotic and meiotic analyses

Root tips and microsporocytes were collected and prepared following Peffley and Mangum (1990). Chromosome counts and karyotypes were made from mitotic metaphases of 16 F_1BC_3 plants following Peffley and Currah (1988). Chromosome arm ratio was calculated with the length of short arm divided by the length of whole chromosome. Relative chromosome length was calculated as the length of a chromosome divided by the sum of the length of all chromosomes in one cell. Microsporocytes were analyzed at first prophase, metaphase, anaphase, and telophase.

Probe labeling

All DNAs used for probes were labeled with biotin-21-dUTP (Nick-translation kit, ClonTech, Palo Alto, Calif., USA). Probes employed in this study included labeled genomic DNAs of *A. cepa* 'New Mexico Sunlite', *A. fistulosum* 'Ishikura', and a 376-bp highly repetitive DNA sequence of *A. fistulosum* (Irifune et al. 1995). Genomic DNA was extracted from young leaves of *A. fistulosum* 'Ishikura' and *A. cepa* 'New Mexico Sunlite' following Wettashinghe and Peffley (1998). The 376-bp highly repeated DNA clone (pAfi105) was provided by Irifune (Hiroshima University, Japan).

In situ hybridization protocols

Root tips used for ISH were digested in 15% pectinase and 5% cellulase (Sigma, St. Louis, USA) for 2 h at 37°C in 0.1 M sodium citrate buffer, pH 4, and squashed in acetocarmine.

ISH was carried out with different hybridization mixtures: genomic in situ hybridization (GISH) where (1) *A. cepa* chromosomes were hybridized with labeled *A. cepa* or *A. fistulosum* genomic DNA respectively; (2) *A. fistulosum* chromosomes were hybridized with labeled *A. cepa* and unlabeled *A. fistulosum* genomic DNAs; (3) chromosomes of the interspecific F_1 hybrid '8273', and F_1BC_3 plants 951027-54 and 951027-97 were hybridized with labeled *A. fistulosum* genomic DNA and blocked with unlabeled *A. cepa* genomic DNA; and (4) fluorescence in situ hybridization (FISH) was done by hybridizing chromosomes of *A. cepa*, *A. fistulosum*, F_1, and F_1BC_3 with labeled pAfi105.

ISH was carried out following Ricroch et al. (1992) with the following modifications: the hybridization mixture for each slide contained 15 µl of formamide, 3 µl of 20× SSC, 2.5 µl of herring sperm DNA (500 µg/ml), 3.5 µl of 50%DS, and 6 µl of probe (20 ng/µl). When blocking-DNA was used for hybridization, the genomic DNA was autoclaved for 5 min, resulting in genomic DNA fragments about 100–500 bp, and added at a concentration of 0.3 µg/µl. Hybridization was carried out in a moist chamber at 37°C for 16 h. Avidin/antibody amplification was repeated once. Slides were counterstained with 0.1% propidium iodide (PI) and 0.01% 4,6-diamidino-2-phenylindole (DAPI) in VectaShield solution (Vector, Burlingame, Calif.), and viewed with UV light at 436 nm allowing simultaneous observation of the hybridized probe (yellow-green) and total DNA (orange). Visualization and photography were done with an Olympus Vanox Epi-fluorescent microscope using Kodak 400 film exposed at E.I. 800, reciprocity 4, and in dark field.

Results

Cytology of F_1BC_3 plants

Sixteen F_1BC_3 plants were analyzed for ploidy level and isozymes. Fifteen of the 16 plants were diploid with 16 chromosomes; eight exhibited *A. fistulosum* isozyme alleles (Table 1). Of these eight, three individuals were selected for further analyses. Plant 951027-75 had chromosome numbers of 16–28 in 53 cells observed (Table 2). Meiotic analysis of 951027-75 revealed eight bivalents at metaphase-I, and aberrant chromosomal behavior such as bridges, laggards, and micronuclei. This plant morphologically resembled an F_1 hybrid with a long neck and slight bulbing, yet had 92.9% pollen stainability and produced six seeds. Plant 951027-97 resembled the F_1 hybrid, in that it had a foliage morphology intermediate to *A. cepa* and *A. fistulosum*, was non-bulbing and pollen-sterile. Analyses revealed recombinant chromosomes. When the relative chromosome length of each chromosome was calculated and compared to the *A. cepa* 'New Mexico Sunlite' chromosomes reported by Ricroch et al. (1992), the longest chromosomes of this plant were much longer than the standard *A. cepa* chromosomes (Table 3). Additionally, one NOR chromosome (No. 11) had a relative length (5.39) close to the 5F NOR chromosome (5.93) of *A. fistulosum* 'Ishikura', but the arm ratio was nearly metacentric (0.428), quite different from the latter (0.228). Also the satellite or secondary constriction was much larger than that of the normal *A. fistulosum* NOR chromosome.

Table 1 Chromosome numbers and some characters of recombinant F_1BC_3 plants with *A. fistulosum* isozyme alleles

Plant #	No. of mitotic chromosomes	Isozyme alleles[a]			Bulbing	Fertility	
		ADH	PGI	EST		Pollen	Seeds
951027-21	16			f/c	Bulbing		
951027-39	16	f/c			Bulbing	90.1%	14
951027-43	16	f/c			Bulbing		
951027-57	16		f/c		Bulbing		
951027-71	16	f/c	f/c		Bulbing		
951027-75	16–28			f/c	Non-bulbing	92.9%	6
951027-96	16		f/c		Bulbing		
951027-97	16	f/c	f/c	f/c	Non-bulbing	0	0
A. cepa	16	c/c	c/c	c/c	Bulbing	94.0%	202
A. fistulosum	16	f/f	f/f	f/f	Non-bulbing	94.9%	159

[a] f=*A. fistulosum* isozyme alleles, c=*A. cepa* isozyme alleles

Table 2 Mitotic and meiotic analysis of F_1BC_3 plant 951027-75

Chromosomal configurations	No. of cells observed	% Total cells
Mitotic chromosome numbers		
16	9	19.15
17	18	38.30
18	3	6.38
19	5	10.64
20	1	2.13
21	3	6.38
22–28	8	17.02
Diakinesis/metaphase-I		
8 oll	2	8.33
8 oll	4	16.44
8 oll	2	8.33
8 oll	1	4.17
7 oll+1 cll	11	45.83
6 oll+2 cll	3	12.5
5 oll+3 cll	2	8.33

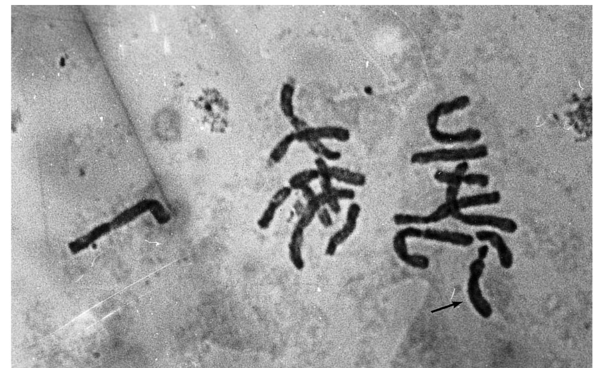

Fig. 1 Karyotype of F_1BC_3 plant 951027-54

Plant 951027-54 exhibited recombinant morphological characters, similar to *A. cepa* in bulbing and neck growth, yet had *A. fistulosum*-type foliage, and both *A. fistulosum* and *A. cepa* PGI isozyme alleles. Twelve chromosomes could be matched into six pairs of homologous chromosomes, but four chromosomes could not be paired (Fig. 1). One NOR chromosome (arrow) resembled *A. fistulosum* chromosome 5 (5F) but had an arm with a satellite larger than the 5F chromosome.

ISH patterns in parental plants and the F_1 hybrid

When biotin-labeled *A. cepa* genomic DNA was hybridized to *A. cepa* chromosomes, the telomeres fluoresced weakly on each arm of all 16 chromosomes. An average of four weak interstitial sites were observed in each of six cells with no consistent pattern. In one cell, five interstitial spots were observed on the long arms of five chromosomes, and 15 on the short arms of nine chromosomes. In some cells no interstitial sites were detected.

When biotin-labeled *A. cepa* genomic DNA was hybridized to *A. fistulosum* chromosomes, the entire chromosomes fluoresced, with the exception of one NOR telomere.

When biotin-labeled *A. fistulosum* genomic DNA was hybridized to *A. cepa* chromosomes, weak signals were detected (Fig. 2a). In three cells with 16 chromosomes, an average of 21 telomeric sites per cell fluoresced weakly.

When biotin-labeled *A. fistulosum* genomic DNA was hybridized to *A. fistulosum* chromosomes, the entire length and each telomere of all chromosomes fluoresced; the intensity of telomeric sites was always much stronger than when labeled *A. fistulosum* genomic DNA was hybridized onto *A. cepa* chromosomes (Fig. 2b).

GISH performed on chromosomes of F_1 hybrid ‘8273’ with labeled *A. fistulosum* genomic DNA probe and blocked with *A. cepa* genomic DNA revealed 8 of 16 chromosomes with strongly fluorescing telomeres (Fig. 2c). Based upon hybridization sites of the parental patterns, *A. cepa* genomic DNA blocked the interstitial hybridization of labeled *A. fistulosum* DNA to the *A. fistulosum* chromosomes but not at all the telomeres. These eight chromosomes are considered to be *A. fistulosum*. Among these eight chromosomes only the satellite re-

Table 3 Chromosome morphology of *A. cepa* 'New Mexico Sunlite'[a], *A. fistulosum* 'Ishikura'[b], and two F_1BC_3 plants, 951027-54 and 951027-97, and telomeric GISH sites (average of six cells). * Indicates one telomeric fluorescing region; ** indicates two telomeric fluorescing regions

Genotype	Chromosome number	Arm ratio (SA/SA+LA)	Relative chromosome length (%)	Genotype	Chromosome number	Arm ratio (SA/SA+LA)	Relative chromosome length (%)
New Mexico Sunlite	1	0.463±0.058	7.61±0.31	Ishikura	1	0.463±0.005	7.94±0.13
	2	0.400±0.037	7.16±0.11		2	0.373±0.006	7.42±0.09
	3	0.399±0.034	6.48±0.20		3	0.409±0.011	6.91±0.05
	4	0.396±0.033	6.18±0.26		4	0.428±0.008	6.09±0.07
	5	0.459±0.019	6.07±0.26		5	0.228±0.011	5.79±0.07
	6	0.262±0.033	5.62±0.34		6	0.470±0.005	5.75±0.08
	7	0.466±0.022	5.48±0.58		7	0.393±0.010	5.20±0.06
	8	0.390±0.020	4.91±0.06		8	0.463±0.005	4.92±0.07
951027-54	1	0.450±0.053	8.67±0.80	951027-97	1	0.470±0.035	9.77±0.35
	2*	0.414±0.029	7.96±0.59		2	0.373±0.037	8.52±0.13
	3	0.411±0.030	7.83±0.57		3	0.403±0.061	8.22±0.70
	4	0.310±0.025	7.52±0.33		4	0.440±0.014	7.87±0.77
	5	0.374±0.086	7.27±0.73		5	0.457±0.098	7.60±0.85
	6	0.456±0.015	7.37±0.92		6	0.317±0.024	6.64±0.30
	7	0.291±0.052	6.85±0.11		7**	0.455±0.076	6.48±0.28
	8	0.448±0.065	6.59±0.30		8**	0.364±0.055	6.25±0.24
	9*	0.395±0.078	6.09±0.26		9	0.447±0.019	6.38±0.29
	10	0.280±0.017	5.95±0.10		10	0.431±0.004	6.11±0.25
	11*	0.356±0.014	5.55±0.52		11*	0.428±0.011	5.39±0.98
	12	0.269±0.015	5.12±0.26		12**	0.497±0.044	4.92±0.18
	13	0.343±0.044	4.87±0.37		13**	0.371±0.033	4.75±0.16
	14	0.328±0.063	4.61±0.29		14**	0.460±0.029	4.78±0.08
	15	0.467±0.039	4.56±0.12		15*	0.462±0.017	4.41±0.58
	16	0.446±0.051	3.99±0.54		16*	0.427±0.028	3.18±0.34

[a] Ricroch et al. 1992 [b] Peffley and Currah 1988

gion of the NOR chromosome was non-fluorescing. Some weak interstitial and telomere signals appeared on the other chromosomes but were distinguishable from those eight stronger fluorescing chromosomes.

The biotin-labeled pAfi105 hybridized to the telomeres of both *A. cepa* and *A. fistulosum* chromosomes, but hybridization sites were much more intense in *A. fistulosum* than in *A. cepa* (Fig. 2d and e). The fluorescing signals were consistently weak and difficult to record in *A. cepa* chromosomes. pAfi105 which hybridized to chromosomes of the interspecific F_1 8273 revealed intense FISH sites at the telomeres of eight chromosomes (Fig. 2f). Similar telomeric hybridization patterns were observed when hybridized with labeled *A. fistulosum* genomic DNA.

ISH patterns in F_1BC_3 plants

When blocked with *A. cepa* genomic DNA and hybridized with biotin-labeled *A. fistulosum* DNA three chromosomes of 951027-54 exhibited strong signals at the telomeric regions (Fig. 2g). Average ratios of the short arm/whole chromosome length of these three chromosomes were 0.414, 0.395 and 0.356, respectively, these from six cells (Table 3). Signals on these chromosomes indicate that the telomeric regions are of *A. fistulosum* origin, yet the morphology of these chromosomes were different from the *A. fistulosum* 'Ishikura' chromosomes reported previously (Peffley and Currah 1988). Chromosome 2 was the second longest and longer than the longest *A. cepa* chromosome reported by Ricroch et al. (1992). It had similar relative chromosome length (7.96%) to 1F (7.94%) but a shorter SA/LA ratio (0.414) than 1F (0.463) Chromosome 9 had the same relative chromosome length as 4F (6.09) but a smaller arm ratio (0.395) than the latter (0.428). On each of these chromosomes only one telomere was flourescing, indicating that one *A. cepa* telomeric region had been substituted by one *A. fistulosum* telomeric region. One telomere on each of these three chromosomes was non-fluorescing, indicating that these telomeres might be from *A. cepa*.

A mitotic spread of 951027-97 revealed both long chromosomes and very short chromosomes (Fig. 2i). One NOR chromosome (arrow) had larger satellites than 5F in *A. fistulosum*. GISH analyses of 951027-97 revealed eight chromosomes with fluorescing telomeres (Fig. 2h). Five of these chromosomes had two telomeres that fluoresced, while three chromosomes (including the NOR, arrow) exhibited only one telomeric hybridization site. One telomere of NOR chromosome 11 fluoresced on the longer arm. Chromosomes 15 and 16 also had fluorescing telomeric sites on the longer arms.

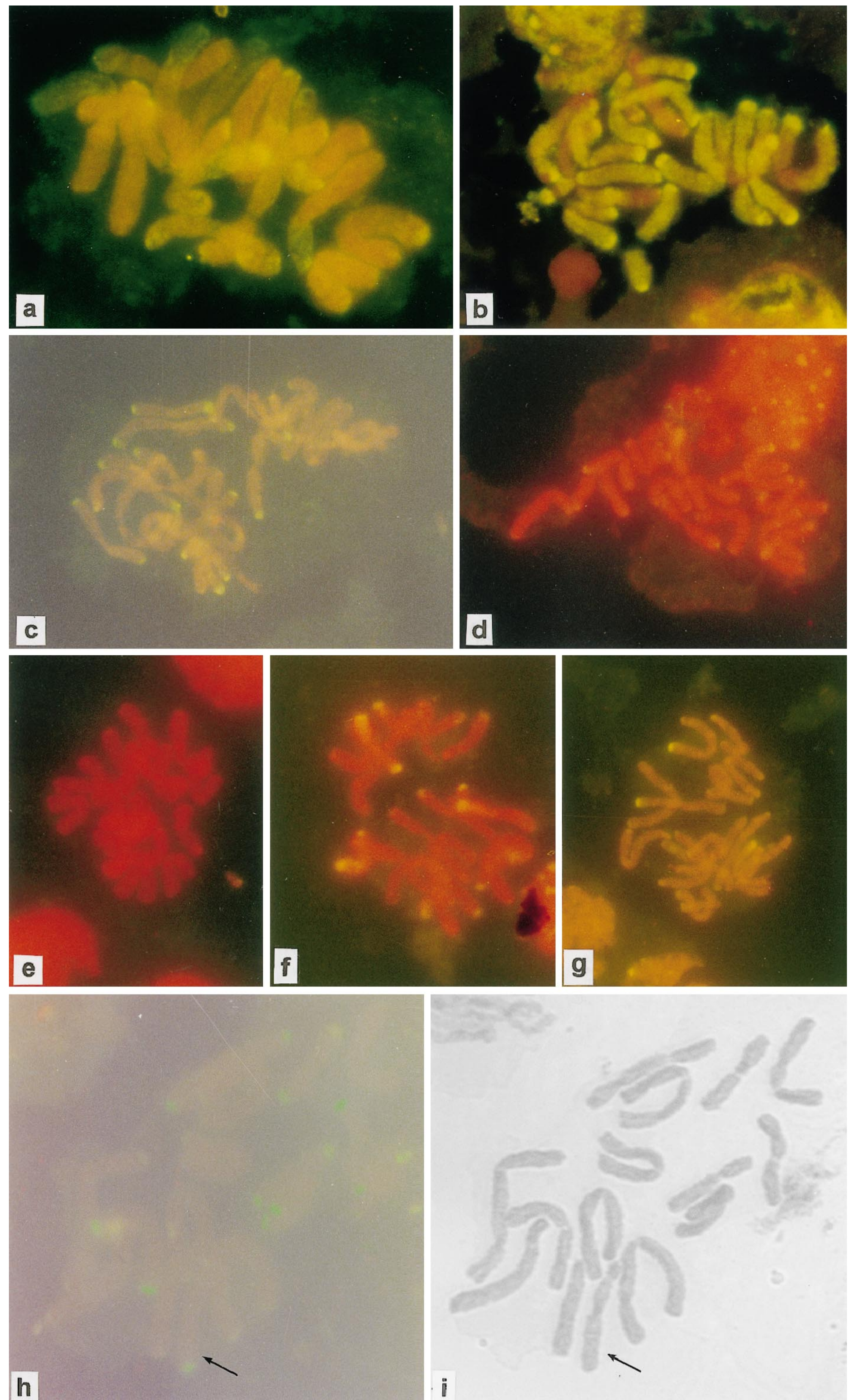

Fig. 2a–i Photographs of FISH and GISH with biotin-labeled genomic DNA and a 376-bp repetitive DNA sequence: **a** *A. cepa* chromosomes hybridized with biotin-labeled *A. fistulosum* genomic DNA; **b** *A. fistulosum* chromosomes hybridized with biotin-labeled *A. fistulosum* genomic DNA; **c** *A. fistulosum*×*A. cepa* F_1 chromosomes hybridized with biotin-labeled *A. fistulosum* genomic DNA and blocked with unlabeled *A. cepa* ge-nomic DNA; **d** *A. fistulosum* chromosomes hybridized with biotin-labeled 376-bp repetitive DNA; **e** *A. cepa* chromosomes hybridized with biotin-labeled 376-bp repetitive DNA; **f** *A. fistulosum*×*A. cepa* F_1 chromosomes hybridized with biotin-labeled 376-bp repetitive DNA; **g** F_1BC_3 plant 951027-54 chromosomes hybridized with biotin-labeled *A. fistulosum* genomic DNA and blocked with unlabeled *A. cepa* genomic DNA; **h** F_1BC_3 plant 951027-97 hybridized with biotin-labeled *A. fustulosum* genomic DNA and blocked with unlabeled *A. cepa* DNA; **I** F_1BC_3 plant 951027-97 mitotic chromosomes before GISH in **h**

Discussion

The distribution of constitutive heterochromatin as revealed by C-banding has been observed at both telomeres of the *A. cepa* and *A. fistulosum* chromosomes (Kalkman 1984; Narayan 1988). GISH and FISH results in this study revealed much stronger hybridization sites at telomeres on chromosomes of *A. fistulosum*, with relatively weaker ones on *A. cepa* telomeres, when hybridized with *A. fistulosum* genomic DNA. Similar GISH patterns were observed in the interspecific hybrids reported by Khrustaleva and Kik (1998). This may indicate that the heterochromatin distribution in these two species differs at the telomeres. Highly repetitive DNA might have been generated by the amplification and transposition of small segments of DNA (Flavel 1982). Repetitive DNAs are useful markers of individual chromosomes because they can be used for in situ hybridization to recognize chromosomes (Flavel et al. 1987). The 376-bp repetitive DNA sequence *from A. fistulosum* employed in this study was able both to distinguish chromosomes of *A. cepa* and *A. fistulosum* and to track chromosomal segments introgressed from the *A. fistulosum* genome.

Repetitive DNA sequences of *A. cepa* and *A. fistulosum* have been reported to hybridize to telomeres in *Allium* crops (Barnes et al. 1985; Fuchs et al. 1995; Narayan 1988; Ricroch et al. 1992). The GISH results in this study revealed similar patterns at the telomere regions, though *A. fistulosum* might have more copies of this repetitive DNA sequence than *A. cepa*. The strong signals detected at the telomeres of *A. fistulosum* might indicate that telomeres may have an additional function instead of being just a pool of junk heterochromatin. Fuchs et al. (1995) hybridized repetitive DNA sequences of *A. cepa* and *A. fistulosum* to *Allium* telomeric regions, suggesting a possible role of telomeric stabilization by repetitive DNA sequences. DNA variation is largely due to repetitive DNAs out of which heterochromatin is composed. The telomeres may be regions that cause major specific differences between *A. cepa* and *A. fistulosum*.

Interstitial-site hybridization was not consistent in this study because we could not identify specific interstitial patterns for each individual chromosome. This appears to be a common occurrence in ISH studies of *Allium* (Fuchs et al. (1996). The evidence produced by these authors suggests that the cell wall and cytoplasm structure in plant cells possibly cover the chromosomes, preventing sufficient hybridization and so obscurring signals.

The F_1BC_3 plant 951027-97 had an NOR chromosome with a satellite larger than the non-recombinant 5F chromosome. Only one telomere of the chromosome fluoresced, the satellite did not fluoresce. This might be due to the fact that the NOR telomere was blocked by *A. cepa* genomic DNA, or else that the telomere was replaced by an *A. cepa* chromosome segment. Translocations involving 5F have been reported by both Peffley (1986) and Ulloa-G et al. (1994). Given high chromosome homoeology between *A. cepa* and *A. fistulosum* genomes (Peffley 1986) and various numbers of bivalents in the interspecific hybrids, certain chromosomes such as 5F might have more fitness to pass on to the advanced generations while the others would be lost, leading to the loss of genes from *A. fistulosum* during introgression. This could explain the difficulty of recovering more *A. fistulosum* traits in the interspecific derivatives or the preponderance of specific *A. fistulosum* traits in the backcrosses. Frequent observation of the 5F chromosome may also lead to the speculation that it could trigger earlier cell division than in *A. cepa*, causing unstable chromosomal disjunction. A direct result of this is the high sterility of the recombinant plants due to the loss of chromosomal segments.

By the BC_3 generation, 94% of *A. cepa* genetic components is expected in the progeny. However, the results of genomic in situ hybridization showed that eight of the 16 chromosomes of F_1BC_3 plant 951027-97 had 13 strongly fluorescing and presumptive *A. fistulosum* telomeres. This plant is completely sterile (Table 1). The mechanism of the recovery of these chromosomes is not clear; one possibility is this plant was derived from continuous selfing rather than backcrossing. However, some chromosomes are recombined. It will be of great interest to further evaluate the introgressed *A. fistulosum* DNA in terms of its stability in the following generations, its effects on chromosomal behavior, and on morphological expression.

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