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The fate of recombinant chromosomes and genome interaction in *Nicotiana* asymmetric somatic hybrids and their sexual progeny

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Abstract Genomic in-situ hybridization (GISH) was used to monitor the behaviour of parental genomes, and the fate of intergenomic chromosome translocations, through meiosis of plants regenerated from asymmetric somatic hybrids between *Nicotiana sylvestris* and *N. plumbaginifolia*. Meiotic pairing in the regenerants was exclusively between chromosomes or chromosome segments derived from the same species. Translocation (recombinant) chromosomes contained chromosome segments from both parental species, and were detected at all stages of meiosis. They occasionally paired with respectively homologous segments of *N. sylvestris* or *N. plumbaginifolia* chromosomes. Within hybrid nuclei, the meiotic division of *N. plumbaginifolia* lagged behind that of *N. sylvestris*. However, normal and recombinant chromosomes were eventually incorporated into dyads and tetrads, and the regenerants were partially pollen fertile. Recombinant chromosomes were transmitted through either male or female gametes, and were detected by GISH in sexual progeny obtained on selfing or backcrossing the regenerants to *N. sylvestris*. A new recombinant chromosome in one plant of the first backcross generation provided evidence of further chromosome rearrangements occurring at, or following, meiosis in the original regenerant. This study demonstrates the stable incorporation of chromosome segments from one parental genome of an asymmetric somatic hybrid into another, via intergenomic translocation, and reveals their transmission to subsequent sexual progeny.

Key words *Nicotiana plumbaginifolia* · *N. sylvestris* Asymmetric somatic hybrids · Meiosis · Sexual progeny Intergenomic translocations · In-situ hybridization

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Introduction

Many plant improvement strategies depend on stable incorporation of potentially useful alien genes into a suitable host genome. This can be achieved in several ways, including sexual hybridization, somatic cell fusion, and direct gene transfer. Such alternative routes have different implications for the survival of introduced genes. In sexual and somatic hybrids, alien DNA is transferred as either whole genomes, chromosomes or chromosome segments. The main disadvantages of chromosome addition lines are that they may express unwanted DNA from the donor genome, or be unstable in the monosomic condition. Alternatively, interaction between parental genomes may result in disruption of meiosis in the hybrid or unilateral elimination of parental chromosomes (Krumbiegel and Schieder 1981; Fehér et al. 1992). Small segments of DNA from one species incorporated into the genome of another by chromosome translocations are therefore attractive for the stable introduction of new genes with minimum disruption of the host genome. In asymmetric somatic hybrids, where irradiated protoplasts from one plant species (donor) are fused with non-irradiated protoplasts of another (recipient), the possibility of obtaining intergenomic translocations is maximized. Fractionation of the donor chromosomes with lethal doses of irradiation generates random fragments, which are available for recombination with recipient chromosomes soon after cell fusion (Parokonny et al. 1992b).

Utilization of intergenomic translocations for interspecific gene transfer depends on the provision of a homo(eo)logous pairing partner at meiosis, and subsequent segregation of recombinant chromosomes to the gametes. In some cases, transmission of alien DNA to the sexual progeny of somatic hybrids is detectable by cytogenetic analysis (Famelaer et al. 1990). In others, however, the similar chromosome morphology of parental species permits only the use of indirect methods (e.g., segregation analysis of marker genes; Bates et al. 1987; Hinnisdaels et al. 1991). Intergenomic translocations are often difficult to recognize from chromosome morphology alone (Famelaer

et al. 1990; Hinnisdaels et al. 1991), although they can sometimes be identified by morphological markers such as nucleolus-organizing regions (NORs) (White and Rees 1985), C-banding patterns (De Vries et al. 1987), or in-situ hybridization (Piastuch and Bates 1990; Parokonny et al. 1992 b).

GISH, which utilizes total genomic DNA as a probe, allows chromosomes from different parental origins to be "painted" in different colours in the nuclei of interspecific hybrids (Parokonny et al. 1992a, b; Schwarzacher et al. 1992; Anamthawat-Jónsson et al. 1993; Bailey et al. 1993; Friebe et al. 1993; Kenton et al. 1993; Mukai et al. 1993). Using GISH, we previously detected translocations between the chromosomes of *N. sylvestris* and *N. plumbaginifolia* in their asymmetric somatic hybrids (Parokonny et al. 1992 b). The translocations were characterized in 4 out of 31 plants regenerated from different individual nuclear hybrid colonies. Two of these regenerant plants were subsequently allowed to flower, and either selfed, or backcrossed to wild-type *N. sylvestris*. Here, we report on the behaviour of chromosomes from each of the parental species, and the fate of intergenomic translocation products (hereafter termed "recombinant chromosomes"), during meiosis of the original regenerants and in their sexual progeny.

Materials and methods

Plant material

Asymmetric somatic hybrids between *N. plumbaginifolia* and *N. sylvestris* were obtained by fusion of leaf mesophyll protoplasts from one parent (recipient) with gamma-irradiated mesophyll protoplasts from the other (donor) (Korostash et al. 1991). The genomic constitution of two regenerants (1VC-1 and 10NV-2) selected for further study was described previously (Parokonny et al. 1992 b), and is summarized in Table 1.

Pollen fertility and self compatibility

The original regenerants of 1VC-1 and 10NV-2 were transferred to the greenhouse and allowed to flower under natural conditions of daylight during spring and summer. The percentage of stainable pollen was estimated from 200 pollen grains stained with 1% cotton blue in lactophenol. Flowers were emasculated and isolated from pollinators by enclosing them in empty gelatin capsules. They were either self-pollinated or used as the female parent in backcrosses with wild-type *N. sylvestris*. Growth of pollen in the style, and subsequent fertilization, was examined as described by Owens (1979).

Seed progeny

Pollinations in which fertilization occurred were repeated and seed was allowed to set. Seeds were germinated in sterile culture on MS medium without hormones, containing 1% agar (Murashige and Skoog 1962). Vigorous seedlings were transferred to the greenhouse.

Chromosome preparations

Root-tip metaphases were prepared as described by Parokonny et al. (1992 b). For successful GISH of meiotic pollen mother cells (PMCs), removal of cell walls was necessary. Meiotic PMCs were squeezed from immature anthers into a drop of either 2% aceto-orcein or 45% acetic acid, on slides treated with Vectabond (Vector Laboratories, Peterborough, UK) to aid cell adhesion, and a coverslip was added. The slides were passed briefly through a flame and left at 4 °C in an atmosphere of 45% acetic acid for 30 min to several hours. After breaking the cell walls by light pressure on the coverslip, slides were re-flooded with aceto-orcein (for conventional staining) or 45% acetic acid (for GISH) until the protoplasts floated free of the cell walls. Chromosome spreads were then optimally flattened and coverslips removed in liquid nitrogen.

Genomic in-situ hybridization (GISH)

The methods used for probe preparation and in-situ hybridization were as described by Parokonny et al. (1992b). For use as a probe, total genomic DNA from *N. sylvestris* was mechanically sheared and labelled with biotin-14-dATP by nick translation. Slides were hybridized with 15 µg/ml of biotinylated probe overnight at 37 °C. Post-hybridization washes were in 2×SSC at 42 °C, 50% formamide, 50% 2×SSC at 42 °C and 2×SSC at room temperature. Biotinylated DNA was detected with fluoresceinated avidin, using one amplification with biotinylated anti-avidin D. Unhybridized DNA was visualized

Table 1 Chromosomal constitution of original regenerants and their sexual progeny

Genotype code number	Origin	Number of progeny plants	Total chromosome number	Number of *N. sylvestris* chromosomes	Number of *N. plumbaginifolia* chromosomes	Number of intergenomic translocations
1VC-1	Regenerant	–	41	22[a]	18	1
1VC-1/BC1	Backcross	1	31	22	9	0
1VC-1/BC1	Backcross	3	31	21	9	1
1VC-1/BC1	Backcross	21	32	23	9	0
1VC-1/BC1	Backcross	4	32	22	9	1
1VC-1/BC1	Backcross	3	33	24	9	0
1VC-1/BC1	Backcross	5	33	23	9	1
IVC-1/BC1-9	Backcross	1	33	22	9	2
IVC-1/S1	Self	3	42	22	20	0
IVC-1/S1-3	Self	1	43	22	20	1
10NV-2	Regenerant	–	64	46	16[b]	2
10NV-2/21	Self	1	52	35	15[c]	2

[a] Includes 21 normal-sized *N. sylvestris* chromosomes and one "minichromosome"
[b] Includes nine normal-sized *N. plumbaginifolia* chromosomes, five "minichromosomes" and two intragenomic recombinant chromosomes
[c] Includes 13 normal-sized *N. plumbaginifolia* chromosomes and two "minichromosomes"

Table 2 Chromosome pairing at metaphase-I in orcein-stained PMCs of *N. sylvestris*, *N. plumbaginifolia*, original regenerants and their sexual progeny

Genotype	2n	No. of cells	Mean configurations per cell ± SD[a] (range in parentheses)						Mean no. of chiasmata	
			I	II		III	IV	V	Per cell	Per bivalent
				Rods	Rings					
N. sylvestris	24	20	0	4.9±2.0 (3–7)	7.1±2.0 (4–10)	0	0	0	18.8±1.8	1.6±0.5
N. plumbaginifolia	20	20	0	2.0±1.3 (1–5)	9.1±1.3 (5–10)	0	0	0	19.9±1.4	2.1±0.6
IVC-1	41	14	8.6±2.6 (4–12)	11.0±2.4 (9–14)	4.0±2.7 (1–9)	0.3±0.5 (0–1)	0.2±0.4 (0–1)	0	19.5±3.9	1.0[b]
IVC-1/BC1-9	33	20	10.6±2.2 (8–16)	1.6±1 .2 (0–3)	8.5±1.0 (7–11)	0.1±0.3 (0–1)	0	0	20.6±2.1	2.0±0.2
10NV-2	54	12	14.2±5.4 (6–29)	7.9±3.2 (3–12)	5.9±2.5 (1–10)	0.8±0.8 (0–3)	1.9±1.1 (1–4)	0.3±0.4 (0–1)	29.3±3.0	0.9[b]

[a] I, univalent; II, bivalent; III, trivalent; IV, quadrivalent; V, pentavalent
[b] Estimated from mean chiasma frequency per cell divided by the maximum possible number of bivalents

by staining with 0.5 µg/ml of propidium iodide. Chromatin from both parents was detected by counterstaining with 2 µg/ml of 4,6-diamin-idophenylindole (DAPI).

Results

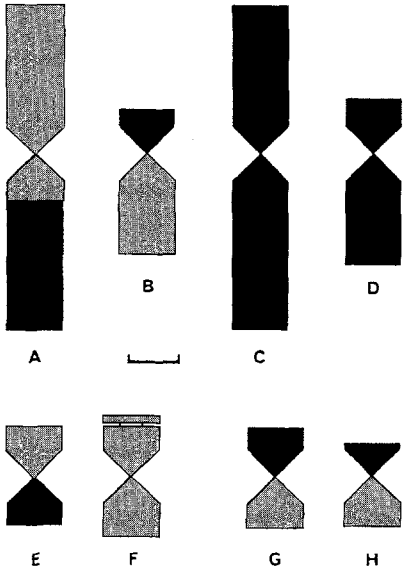
Meiosis in *N. plumbaginifolia* and *N. sylvestris*

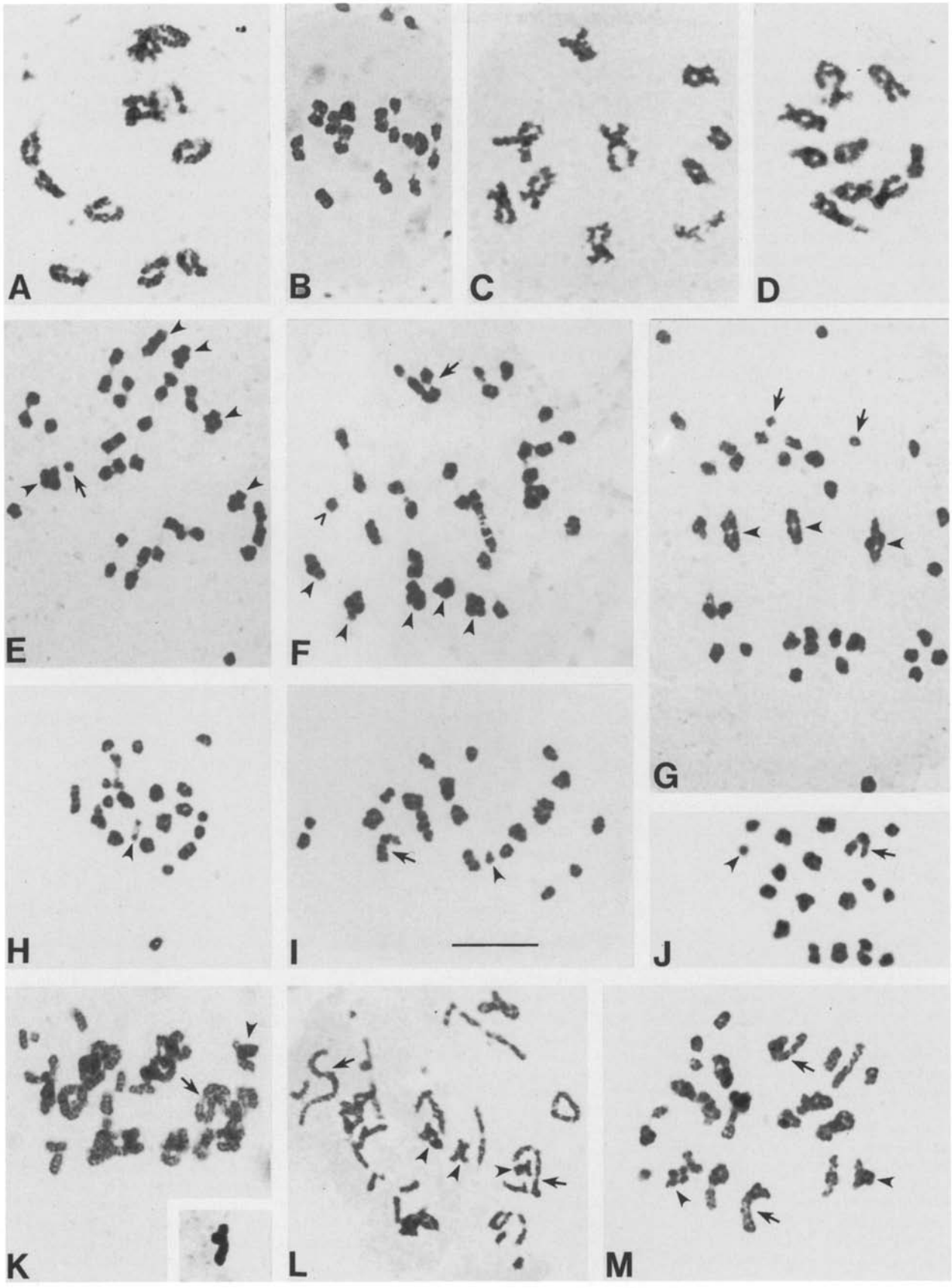
Each of 20 PMCs from *N. sylvestris* (2n=24) and *N. plumbaginifolia* (2n=20) contained a complete complement of bivalents at diakinesis. Chiasma frequency differed between *N. plumbaginifolia* and *N. sylvestris* (Table 2, Fig. 2 A–D), such that the 20 chromosomes of *N. plumbaginifolia* formed a higher proportion of ring (closed) bivalents to rod (open) bivalents than the 24 chromosomes of *N. sylvestris* (95% and 60%, respectively). This difference was reflected in a higher chiasma frequency per bivalent, and relatively more interstitial chiasmata, in *N. plumbaginifolia*. Because bivalents with more than two chiasmata always have one non-terminal chiasma position, only the most-distal chiasma in each chromosome arm was scored when making these comparisons. In *N. sylvestris*, 97% of the most-distal chiasma positions were visibly terminal, compared to only 32% in *N. plumbaginifolia*. Characteristic features of bivalents at diakinesis were confirmed at metaphase I for both species (cf. Fig. 2 A–D).

Chromosome constitution and meiosis of regenerant plants, 1VC-1 and 10NV-2

1VC-1. The somatic chromosome number (2n=41) comprised a hypodiploid chromosome set from each parent. Root-tip metaphases contained 18 chromosomes from *N. plumbaginifolia*, 22 (including one "minichromosome") from *N. sylvestris*, and a very small metacentric resulting from an intergenomic translocation between the chromosomes of these parents (Figs. 1 E, F and 3 A, Table 1). The "minichromosome" was characterized by a NOR in the short arm and a large deletion in the long arm (Figs. 1 F, 3 A).

Fig. 1 A–H Diagrammatic representation of the recombinant chromosomes in 1VC-1, 10NV-2 and 1VC-1/BC1-9. *Solid shading, N. plumbaginifolia* chromatin (red in Figs. 3, 4), *hatched shading, N. sylvestris* chromatin (yellow in Figs. 3, 4). **A–D** 10NV-2, large (**A**) and small (**B**) intergenomic recombinant chromosomes; large (**C**) and small (**D**) intragenomic recombinant chromosomes derived from *N. plumbaginifolia*. **E** and **F** 1VC-1, intergenomic recombinant chromosome (**E**) and *N. sylvestris* "minichromosome" (**F**). **G** and **H** 1VC-1/BC1-9, original (**G**) and new (**H**) intergenomic recombinant chromosomes. Scale bar represents 1 µm





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In orcein-stained PMCs at diakinesis, bivalents derived from *N. plumbaginifolia* could often be identified by their larger size, interstitial chiasmata (Fig. 2 E, F) and terminal centromeres (Fig. 2 E) the latter being especially conspicuous at anaphase-I (Fig. 2 G). However, the parental origin of all configurations was by no means unequivocal. Conversely, in-situ hybridization with biotinylated total genomic DNA from *N. sylvestris* immediately identified all *N. sylvestris* chromosomes by their yellow fluorescence, while *N. plumbaginifolia* chromosomes fluoresced red with the counterstain, propidium iodide (Figs. 3 B, 4 F). It was then clear that a maximum of ten bivalents and two univalents per PMC were derived from *N. sylvestris*, with a maximum of eight bivalents and two univalents from *N. plumbaginifolia*. The two *N. plumbaginifolia* univalents differed in size (Fig. 4 F), suggesting that they were non-homologues. No bivalent involved pairing between interspecific combinations of normal-sized chromosomes.

Although overall chiasma frequencies per cell were very similar for 1VC-1 and the parental species (Table 2), the greater number of chromosomes in 1VC-1 (a hypo-allotetraploid) resulted in a relatively-lower chiasma frequency per bivalent. The number of chiasmata per bivalent was variable for PMCs within and between anthers, and up to 12 chromosomes were univalent (Table 2). Bivalents with interstitial chiasmata were confirmed, by their red fluorescence after GISH, to be derived from *N. plumbaginifolia* (Fig. 4 F).

Both the recombinant chromosome and the "minichromosome" were smaller than the standard chromosomes (Fig. 2 E, F, G), but could be distinguished from one another only after GISH (Figs 3 A, 4 A). In 9 out of 14 orcein-stained PMCs, a heteromorphic association (either bivalent, trivalent or quadrivalent) contained one of these small chromosomes (e.g., Fig. 2 F). In the remaining PMCs, and in numerous others that were not analyzed completely, both small chromosomes were univalent. GISH showed that heteromorphic bivalents contained either a standard *N. sylvestris* chromosome and the "minichromosome" (Fig. 4 F), and/or a standard *N. sylvestris* chromosome and the recombinant. In most PMCs, however, the recombinant chromosome was univalent (Fig. 3 B).

A conspicuous feature of all PMCs was the delayed separation of some bivalents until late in anaphase-I (Fig. 2 G). After GISH, it was clear from their red fluorescence that these were derived from *N. plumbaginifolia* (Fig. 3 C). The division of *N. plumbaginifolia* chromosomes was also retarded at anaphase-II. However, by telophase-I or telophase-II, most *N. plumbaginifolia* chromosomes had been incorporated into a dyad or tetrad nucleus (Fig. 3 D, F). Univalents from both parental origins usually migrated to one of the poles without dividing mitotically. The recombinant chromosome, distinguishable by its dual red and yellow fluorescence, was observed undivided in dyads and tetrads (e.g., Fig. 3 C, E).

At telophase-I and telophase-II, *N. sylvestris* chromosomes occupied a conspicuous domain in dyad or tetrad nuclei (Fig. 3 D, E, F). Bivalents derived from the two species also tended to be localized within individual sectors of two-dimensional chromosome spreads at diakinesis, in both orcein-stained (Fig. 2 E, F) and GISH preparations (Figs. 3 B, 4 F).

Thirty-eight percent of pollen grains in 1VC-1 were stainable and well-filled. Pollen germination, tube elongation and fertilization were observed in selfs and in backcrosses to *N. sylvestris*. Copious seed was set which was highly germinable.

10NV-2. Root-tip nuclei of the original regenerant contained 64 chromosomes. These comprised 46 from *N. sylvestris*, 14 (including five "minichromosomes") from *N. plumbaginifolia* and four recombinant chromosomes (Table 1). Of the recombinants, two originated from intergenomic translocations (Figs. 1 A, B and 3 G, H) and two re-

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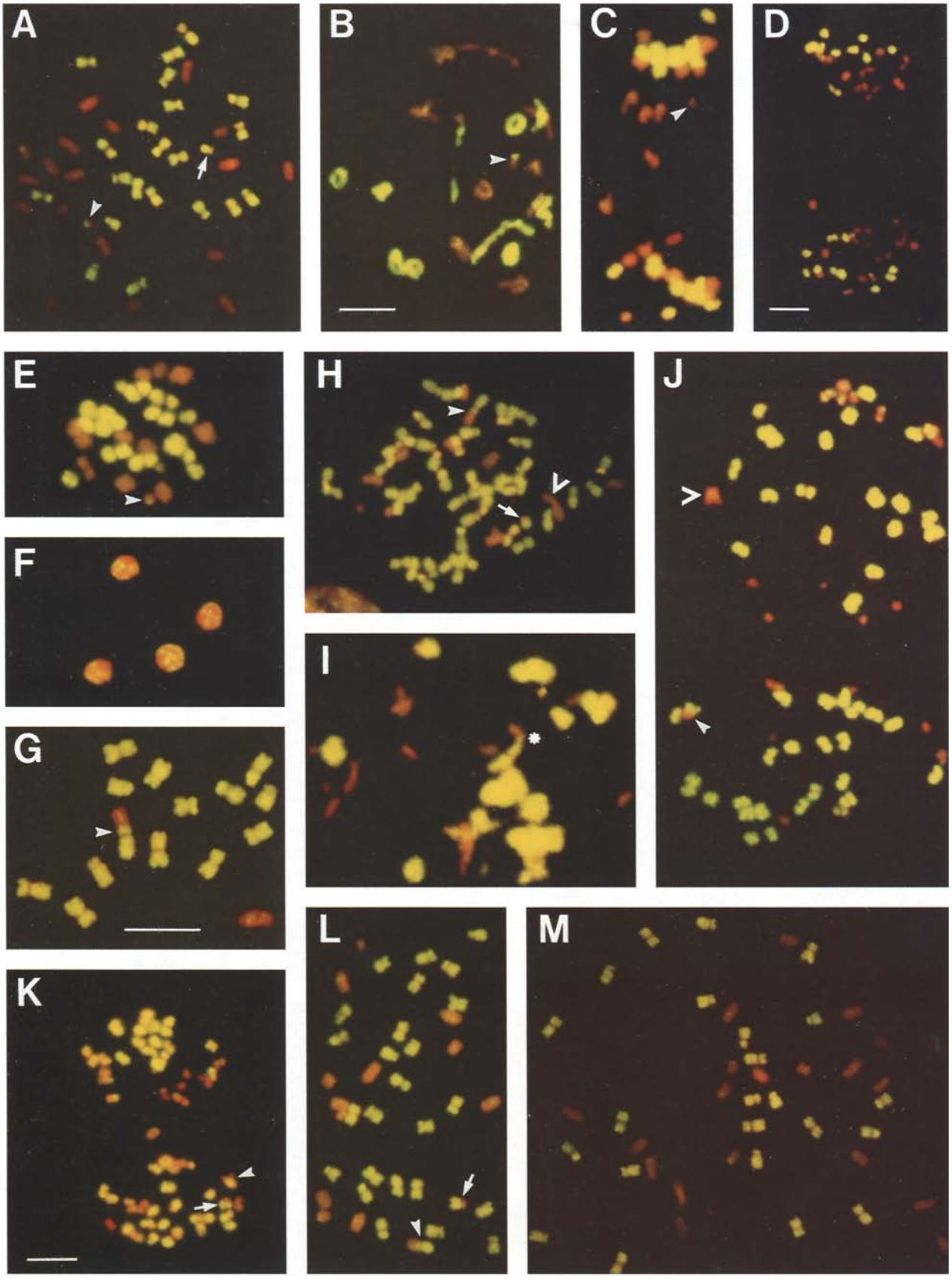
Fig. 2 A–M Orcein-stained preparations of meiosis in PMCs of parental species, original regenerants and their sexual progeny. *N. sylvestris*, early diakinesis (**A**) and metaphase-I (**B**), showing 12 bivalents with terminal chiasmata. *N. plumbaginifolia*, early diakinesis (**C**) and late diakinesis (**D**), showing proximal, interstitial and terminal chiasmata. **E–G** 1VC-1, metaphase I (**E**): *arrow,* small univalent chromosome, *arrowheads*, bivalents with interstitial chiasmata; metaphase-I (**F**): *arrow,* quadrivalent containing a small chromosome, *arrowheads,* bivalents with interstitial chiasmata, *open arrowhead,* small univalent; anaphase-I (**G**): *arrowheads,* lagging bivalents, *arrows,* two small chromosomes. **H–J** 1VC-1/BC1-9, metaphase-I (**H**): *arrowhead,* small bivalent, putatively between two intergenomic recombinant chromosomes (cf. Fig. 1G and H); metaphase I (**I, J**): *arrow,* heteromorphic trivalent containing a small chromosome, *arrowhead,* small univalent. **K, L** 10NV-2, metaphase-I (**K**): *arrowhead,* heteromorphic bivalent between a metacentric and a subtelocentric chromosome, *arrow,* quadrivalent; early diakinesis (**L**): *arrows,* quadrivalents, *arrowheads* putative *N. plumbaginifolia* bivalents. **M** 10NV-2/S1, diakinesis: *arrowheads,* putative *N. plumbaginifolia* bivalents, *arrows,* trivalents. Scale bar represents 10 μm

Fig. 3 A–M GISH of *N. sylvestris* DNA to somatic and meiotic nuclei of original regenerants and their sexual progeny. *N. sylvestris* chromatin fluoresces yellow and *N. plumbaginifolia* chromatin, red. **A–E** 1VC-1, root tip metaphase (**A**), diakinesis (**B**), meiotic anaphase-I (**C**), meiotic telophase-I (**D**) and meiotic dyad nucleus (**E**): *arrow, N. sylvestris* "minichromosome" (cf. Fig. 1 F), *arrowhead,* recombinant chromosome (cf. Fig. 1 E). **F** 1VC-1, post-meiotic tetrads (each nucleus has a red and a yellow sector). **G–K** 10NV-2, root-tip metaphases (**G, H**), diakinesis (**I**) and late meiotic anaphase-I (**J, K**): *arrowhead,* large intergenomic recombinant chromosome (cf. Fig. 1 A), open *arrowhead,* large intragenomic recombinant chromosome derived from *N. plumbaginifolia* (cf. Fig. 1 C), *arrow,* small intergenomic recombinant chromosome (cf. Fig. 1 B), *asterisk,* heteromorphic bivalent between the large intergenomic recombinant chromosome (cf. Fig. 1A) and an normal-sized *N. sylvestris* chromosome. **L** 10NV-2/S1, partial metaphase from a root tip: *arrowhead,* large intergenomic recombinant chromosome (cf. Fig. 1 A), *arrow*, small intergenomic recombinant chromosome (cf. Fig. 1 B), both intragenomic recombinant chromosomes (cf. Fig. 1 C and D) are lacking. **M** 1VC-1/S1-1, root-tip metaphase, intergenomic recombinant chromosome (cf. Fig. 1 E) and *N. sylvestris* "minichromosome" (cf. Fig. 1 F) are lacking. Scale bars represent 10 μm. Scale bar in **D** also refers to **F**. Scale bar in **B** refers to remaining photographs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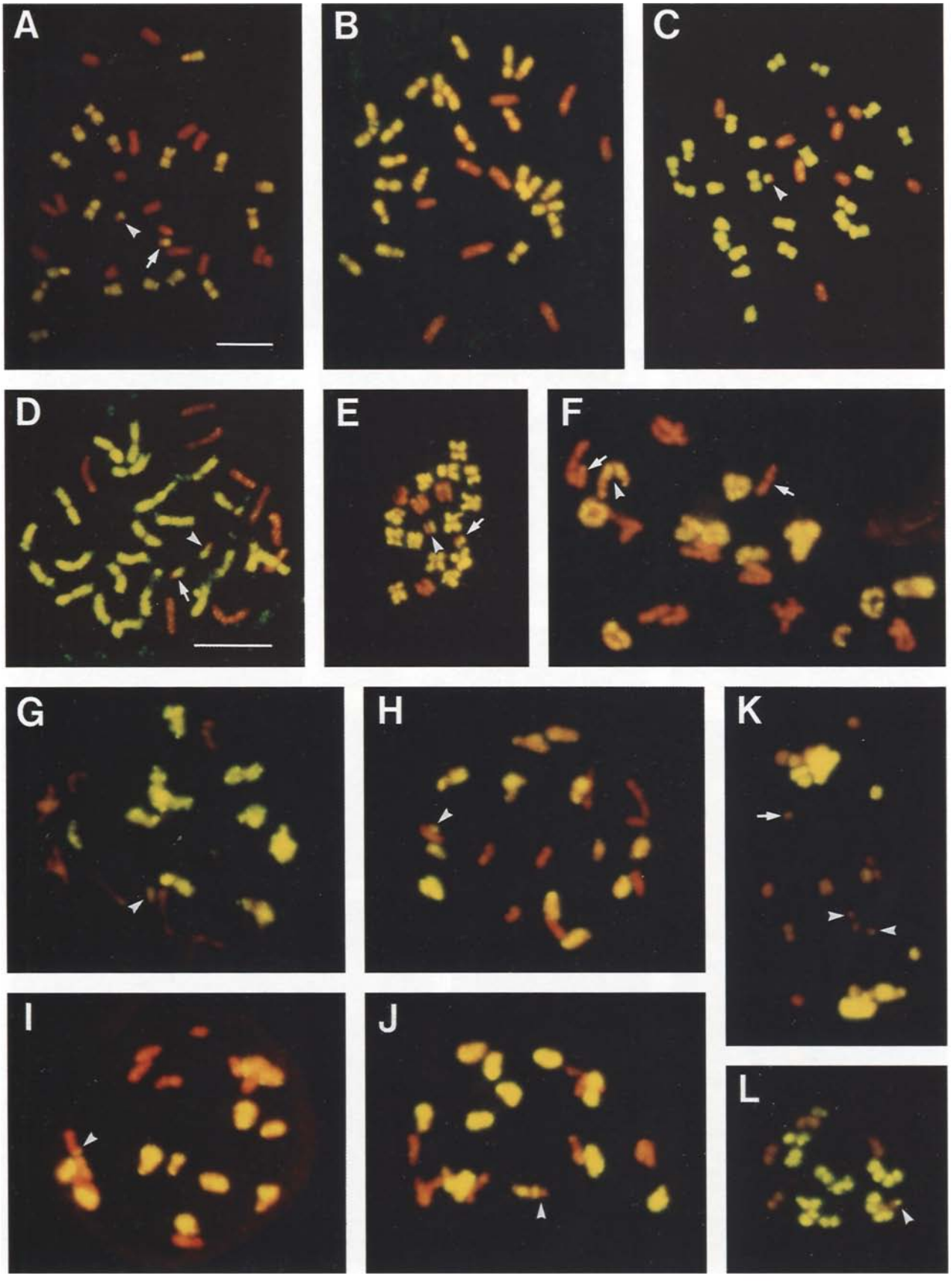
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sulted from intragenomic structural rearrangements among *N. plumbaginifolia* chromosomes (Figs 1 C, D, 3 H).

Orcein-stained PMCs of 10NV-2 at diakinesis contained 54 chromosomes, including at least two "minichromosomes". Multivalents, up to a maximum of four quadrivalents, were present in all PMCs (Fig. 2 K, L, Table 2). In GISH preparations, these multiples were identified as being derived from *N. sylvestris* (Fig. 3 I). Autosyndetic bivalents were formed between chromosomes within complements derived from *N. plumbaginifolia* and *N. sylvestris*, respectively. Normal chromosomes and "minichromosomes" derived from *N. plumbaginifolia* formed heteromorphic bivalents (data not shown). A second type of heteromorphic bivalent, between a metacentric and a subtelocentric (Fig. 2 K), involved the large intergenomic recombinant chromosome and a standard chromosome from *N. sylvestris* (Fig. 3 I). The large intragenomic recombinant chromosome derived from *N. plumbaginifolia* (Fig. 1 C) was seen only as a univalent. Each of the intergenomic recombinants (Fig. 1 A, B), and the large intragenomic recombinant (Fig. 1 C), could be incorporated into dyad nuclei (Fig. 3 J, K). As in 1VC-1, migration of *N. plumbaginifolia* chromosomes to the poles was delayed relative to *N. sylvestris* chromosomes at first and second anaphase, resulting in a separate distribution of chromosomes derived from the two parents in telophase nuclei (Fig. 3 J, K).

Vegetative growth and floral initiation of 10NV-2 was slow, compared to the parental species and 1VC-1. Anthers contained a mean of 50% stainable pollen. Germination, pollen tube growth and fertilization were observed in self pollinations and backcrosses to *N. sylvestris*, using either the hybrid or *N. sylvestris* as the female parent. Capsules were readily set, but most of the seed failed to develop to maturity. Only one seedling, resulting from a self pollination, was recovered.

Fig. 4 A–L GISH of *N. sylvestris* DNA to somatic and meiotic nuclei of original regenerant 1VC-1 and its sexual progeny. *N. sylvestris* chromatin fluoresces yellow and *N. plumbaginifolia* chromatin, red. **A** 1VC-1/S1-3, root-tip metaphase: *arrow*, *N. sylvestris* "minichromosome" (cf. Fig. 1 F), *arrowhead*, original intergenomic recombinant chromosome (cf. Fig. 1 E). **B–E** Backcross progeny of 1VC-1, root-tip metaphases of 1VC-1/BC1-4 (**B**), 1VC-1/BC1-2 (**C**) and 1VC-1/BC1-9 (**D, E**): *arrowhead*, original intergenomic recombinant chromosome (cf. Fig. 1 E or G), *arrow*, new intergenomic recombinant chromosome (cf. Fig. 1 H). **F** 1VC-1, diakinesis: *arrowhead*, heteromorphic bivalent between a normal-sized *N. sylvestris* chromosome and a "minichromosome", *arrows*, monosomic *N. plumbaginifolia* univalents. **G–J** 1VC-1/BC1-9, diakinesis, *arrowhead*, intergenomic recombinant chromosome which is either univalent (**G, H**) or pairs with a normal chromosome from *N. plumbaginifolia* (**I**) or *N. sylvestris* (**J**). **K** 1VC-1/BC1-9, meiotic telophase I, most *N. plumbaginifolia* chromosomes (red) are lagging: *arrowheads*, dividing recombinant chromosomes, *arrow*, undivided recombinant chromosome. **L** 1VC-1/BC1-9, meiotic dyad nucleus with five *N. plumbaginifolia* chromosomes, *arrowhead*, intergenomic recombinant chromosome. Scale bars represent 10 μm. Scale bar in **A** also refers to **B**, **C** and **E–L**

Chromosome constitution and meiosis of sexual progeny

The mitotic chromosome complements of all progeny plants were screened both with conventional Feulgen staining and after GISH with biotinylated total *N. sylvestris* DNA. The chromosome constitution of self and backcross progeny is summarized in Table 1.

Backcross progeny of 1VC-1. Thirty-eight seedlings from three capsules were raised in vitro. Root-tip nuclei of all seedlings contained nine chromosomes from *N. plumbaginifolia* and 21–24 chromosomes from *N. sylvestris* (Table 1). These backcrosses were thus near-allotriploids containing a hypodiploid chromosome set from *N. sylvestris* and a hypohaploid set from *N. plumbaginifolia*. Only 33% of seedlings contained the small recombinant chromosome present in the original regenerant (Fig. 4 B, C). One seedling (1VC-1/BC1-9) contained two recombinant chromosomes, in addition to nine chromosomes from *N. plumbaginifolia* and 22 from *N. sylvestris* (Fig. 4 D), but lacked the "minichromosome" (Fig. 1 F). One recombinant chromosome looked identical to that identified in 1VC-1 (cf. Figs. 1 E, G and 4 D, E), while the other contained a relatively smaller segment of chromatin from *N. plumbaginifolia* (Figs. 1 H and 4 D, E). Both of these recombinant chromosomes were smaller than any standard chromosome of either *N. sylvestris* or *N. plumbaginifolia*, and could thus be recognized in orcein-stained PMCs (Fig. 2 I, J). However, they could be distinguished from one another only after GISH, in favourable preparations (e.g., Fig. 4 D, E).

Meiosis of 1VC-1/BC1-9 was of special interest. In 20 orcein-stained PMCs analyzed at diakinesis, the minimum number of univalents was eight. Most PMCs, however, had nine univalents and a mean of ten bivalents (Table 2). GISH with biotinylated total *N. sylvestris* DNA identified up to ten yellow bivalents from *N. sylvestris* and 8–9 red univalents from *N. plumbaginifolia* (Fig. 4 G-J). *N. plumbaginifolia* univalents often lay apart from *N. sylvestris* bivalents in two-dimensionally-spread nuclei at diakinesis (e.g., Fig. 4 G, H, I).

Most PMCs contained only one recombinant chromosome (Fig. 4 G, H). When both recombinants were present, they were either univalent, or consituted part of a heteromorphic bivalent or trivalent. Such heteromorphic associations were present in 6 out of 20 orcein-stained PMCs (Fig. 2 I, J), two of which also possessed a small univalent. GISH confirmed that heteromorphic bivalents contained one of the recombinant chromosomes and a standard chromosome from either *N. plumbaginifolia* or *N. sylvestris* (Fig. 4 I, J). A minute bivalent, presumed to include both recombinant chromosomes, was present in two orcein-stained PMCs (Fig. 2 H).

At first and second anaphase, there was extensive lagging and bridging of univalents derived from *N. plumbaginifolia* (Fig. 4 K). Although univalents, including the recombinants, could be incorporated into dyad and tetrad nuclei (Fig. 4 L), tetrads often contained one or more micronuclei, and the pollen of 1VC-1/BC1-9 was completely sterile.

Self progeny of 1VC-1. Four seedlings were raised from one seed capsule. Root-tip nuclei from each seedling contained 20 chromosomes from *N. plumbaginifolia* and 22 from *N. sylvestris* (Fig. 3 M, Table 1). The small recombinant chromosome and "minichromosome" from the original regenerant, 1VC-1 (Fig. 1 E, F), were present in root tip metaphases of one seedling only (Fig. 4 A, Table 1).

Self progeny of 10NV-2. Root-tip nuclei of the single seedling raised (10NV-2/S1) had 52 chromosomes (Table 1). These comprised 35 from *N. sylvestris*, 15 (including two "minichromosomes") from *N. plumbaginifolia* and two intergenomic recombinant chromosomes (Fig. 1 A, B). The intragenomic recombinants (Fig. 1 C, D) were both lacking (Fig. 3 L, Table 1). As in 10NV-2, development and floral initiation were slow compared to 1VC-1 and the parental species. Although no GISH analysis was made, multivalents (predominantly trivalents) were observed in orcein-stained PMCs (Fig. 2 M). Bivalents between *N. plumbaginifolia* chromosomes were characterized by interstitial chiasmata and terminal centromeres (Fig. 2 M).

Discussion

GISH improved the genome analysis of *Nicotiana* asymmetric somatic hybrids in three main ways. (1) It allowed the chromosomal constitution of regenerated plants and their sexual progeny to be unequivocally established; (2) it enabled the interaction of parental genomes to be observed at meiosis; (3) it provided information on the potential of intergenomic translocations as vectors of alien DNA.

The recombinant chromosome in 1VC-1 (Fig. 1 E) comprised small segments of chromatin from *N. sylvestris* and *N. plumbaginifolia*. Possibly, it arose via unequal translocation and loss, as reported for a deleted recombinant chromosome in a *Petunia* somatic hybrid (White and Rees 1985). The amount of *N. sylvestris*-derived DNA detectable in the recombinant chromosome by GISH was calculated by comparing the lengths of the translocated segment and normal-sized *N. sylvestris* chromosomes in four root-tip metaphase spreads. The 4C DNA amount of *N. sylvestris* is 11.48 pg (2C=5.74 pg, Narayan 1987). Assuming that 1 pg of DNA contains 965 Mbp (Arumuganathan and Earle 1991), an average *N. sylvestris* chromosome contains 460 Mbp (0.48 pg) of DNA. The length of the *N. sylvestris* segment on the recombinant chromosome is 23% of the average for a standard *N. sylvestris* chromosome, and thus contains about 106 Mbp of DNA.

In 1VC-1, restriction of meiotic pairing to bivalents within each parental genome suggests that 1VC-1 functioned as an allotetraploid. Directed segregation of univalents from each parent, and the maintenance of the chiasma localization characteristic for each species, further indicates that genetic control of meiotic behaviour was exerted by each parental genome, independently of the other.

The formation of either regular or heteromorphic bivalents of *N. plumbaginifolia* origin showed that at least some *N. plumbaginifolia* chromosomes within 10NV-2 were homologous pairs. However, multivalent frequency was well below the maximum of 10–11 quadrivalents expected for the almost complete tetraploid complement derived from *N. sylvestris*. The low number of multiples cannot be explained by lack of chromosome homology, as the tetraploid *N. sylvestris* complement was derived from the fusion of two genetically-identical nuclei. It may, therefore, reflect either the relatively-low overall chiasma frequency (0.9 chiasma per paired chromosome), or chromosome elimination prior to meiosis. Twelve orcein-stained PMCs, analyzed from one anther at diakinesis, contained only 54 chromosomes (Table 2). 10NV-2 was slow to flower in comparison with the hypo-allotetraploid 1VC-1 and diploid parental species, and chromosomes may have been lost during floral initiation or anther development. Replacement of quadrivalents by trivalents in 7 out of 12 PMCs, and a pentavalent in 3 out of 12, suggests that some *N. sylvestris* chromosomes could have been disomic, and others pentasomic, within the PMCs of 10NV-2.

In both 1VC-1 and 10NV-2, normal and recombinant univalents usually moved to one of the poles at anaphase-I without dividing mitotically. They therefore divided normally at anaphase-II and were incorporated into the gametes. Although each parental genome of the hybrids contributed an approximately-balanced number of chromosomes to dyad and tetrad nuclei, gametes may have been aneuploid for particular linkage groups within parental genomes. Such genetic imbalance probably contributed to the relatively-low pollen viability in 1VC-1 and 10NV-2. Fertility levels in both regenerants are comparable to those of *Lycopersicon* somatic hybrids, where numerically-balanced pollen grains were also suggested to be genetically unbalanced (Giddings and Rees 1992).

Gleba et al. (1987) reported spatial separation of genomes derived from *N. plumbaginifolia* and *N. sylvestris* in mitotic metaphases of their somatic hybrids, and proposed the activity of a chimaeric spindle as a possible mechanism. This idea is supported by the differential segregation of *N. plumbaginifolia* and *N. sylvestris* chromosomes observed here in hybrid PMCs. Lagging of *N. plumbaginifolia* chromosomes at first and second anaphase appears to initiate their peripheral distribution in dyad and tetrad nuclei. Lagging may be due to an increase in the duration of meiosis for *N. plumbaginifolia,* consistent with its larger genome size compared to *N. sylvestris* (Bennett 1972; Narayan 1987). Other physical properties, such as terminal centromere position or the organization of centromeric heterochromatin, could also influence the relative efficiency of *N. plumbaginifolia* kinetochores (cf. Vig 1982). However, the latter seems unlikely, since, despite the peripheral location of the *N. plumbaginifolia* genome, *N. sylvestris* (recipient) chromosomes were preferentially eliminated from 10NV-2. An alternative explanation is that specific genetic controls regulated both delayed segregation and localized genome distribution (cf. Anamthawat-Jónsson et al. 1993). Asymmetric somatic hybrids offer an

opportunity to explore genomic adjustment mechanisms following wide hybridization.

Although small, the original recombinant chromosome in 1VC-1 often moved undivided to one of the poles at anaphase-I, surviving in approximately 30% of self and backcross progeny. The new recombinant in 1VC-1/BC1-9 may have been derived from the original one via non-disjunction at anaphase-II, when both chromatids could have been incorporated into the same tetrad nucleus, followed by further rearrangement during in-vitro seed culture. In the triploid backcross progeny, absence of homologous pairing partners for *N. plumbaginifolia* chromosomes increased the likelihood of pairing between recombinant and normal chromosomes. Formation of bivalents between the two recombinant chromosomes in 1VC-1/BC1-9 further increased their chances of survival into subsequent generations. In 10NV-2, two intergenomic recombinant chromosomes were transmitted to the selfed progeny. At least one of these had a partially-homologous pairing partner and is therefore likely to be transferred to further generations.

The present study has shown that intergenomic translocations (1) are readily produced in asymmetric somatic hybrids; (2) are transmitted through male and female meiosis to sexual progeny; (3) are detectable by GISH to a lower size limit of 100 Mbp or less. Detection of the desired genes in translocated chromosome segments may be further improved using sequential in-situ hybridization with probes containing the gene of interest and total genomic DNA from one of the parents (Kenton et al. 1993).

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