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Genomic characterisation and the detection of raspberry chromatin in polyploid *Rubus*

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Abstract This paper reports genomic in situ hybridization (GISH) and fluorescent in situ hybridization (FISH) data for chromosomes of raspberry (*Rubus idaeus* 2n = 2x = 14), blackberry (*Rubus* aggregate, subgenus *Eubatus*. 2n = 2–12x = 14–84) and their allopolyploid derivatives used in fruit breeding programmes. GISH was used to discriminate labelled chromosomes of raspberry origin from those of blackberry origin in allopolyploid hybrid plants. The raspberry chromosomes were labelled by GISH at their centromeres, and 1 chromosome was also labelled over the short arm. In one allopentaploid plant a chromosome carried a terminal signal. Karyotype analysis indicated that this is a blackberry chromosome carrying a raspberry translocation. GISH analysis of an aneuoctaploid blackberry cv 'Aurora' (2n = 8x = 58) showed that both whole and translocated raspberry chromosomes were present. The basic *Rubus* genome has one ribosomal DNA (rDNA) locus, and in all but one case all levels of ploidy had the expected multiples of rDNA loci. Interestingly, in the blackberry cv 'Aurora', there were only six sites, two less than might be predicted from its aneuoctaploid chromosome number. Our results highlight the potential of GISH and FISH for genomic designation, physical mapping and introgression studies in *Rosaceous* fruit crops.

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Introduction

Despite the importance of many members of the Rosaceae family for fruit crops and horticulture, there have been only a few detailed cytogenetic studies. Most species have small chromosomes (1–3 μm in length) which are difficult to analyse cytologically. Thompson (1995) reviewed work describing *Rubus* chromosome counts, and Pool et al. (1981) described the karyotype of *R. idaeus* L. (raspberry) and *R. coreanus* Miquel. The genus *Rubus* has a basic chromosome number of n = x = 7 and a complex polyploid series both naturally and following genome manipulation and crossing. Blackberry (subgenus *Eubatus*, genome designation B), a complex polyploid species aggregate, can occur up to dodecaploid (12x). Raspberry (*R. idaeus*, genome designation R, to primocane fruiting types R′) occurs mainly as a diploid (2n = 2x = 14) in nature and in commercial varieties. The primocane fruiting (PF) forms of raspberry produce fruit at the tips of first-year growth, and this character has been exploited to extend the fruit cropping season (Keep 1988).

In 1993, an EC-funded project was initiated with the aim of producing PF hybrids between raspberry and blackberry that can be grown as a new crop in regions of mild winters (Knight and Rosati 1994). As part of this breeding programme, allopolyploids were made between colchicine-doubled raspberry autoteraploids (2n = 4x = 28, R′R′R′R′) and different blackberry polyploids to make hybrid-berries with the theoretical genomic compositions of R′R′BB, R′R′R′B, R′R′RBB and R′R′BBBB. It was necessary to confirm that the genomic composition of the hybrids was as predicted and to determine whether there were any structural re-arrangements or karyotype changes in the hybrid

breeding material. For this reason genomic in situ hybridisation (GISH, Le et al. 1989; Schwarzacher et al. 1989) was applied to identify raspberry chromosomes, and the data is presented here. Previously, we have reported that GISH can be used to distinguish chromatin from raspberry and blackberry (Lim et al. 1998), but hitherto no detailed descriptions or pictoral data have been presented, nor has the value and application of the method to *Rubus* breeding been demonstrated. In addition, using GISH we have investigated the possible presence of raspberry chromosomes in the blackberry cv 'Aurora' with the putative genomic designation of BBBBBBBB. 'Aurora' was generated by crossing a nonaploid plant (9x, BBBBBBBBR) with a septaploid plant (7x, BBBBBBR). The B genomes may pair and segregate normally in meiosis. However the R genomes, being in single dose, may segregate randomly, be eliminated or pair homoeologously with the B genome. If the latter occurs translocations between the B and R genome chromosomes may take place. Because in situ hybridisation is such a powerful tool to identify chromosomes in plants with small genomes (Jiang and Gill 1994; Jiang et al. 1995) we were able to test these hypotheses.

We also used rDNA probes in fluorescent in situ hybridization (FISH) experiments to examine the number and location of rDNA loci in raspberry and hybrid-berries of different ploidy levels as well as in the blackberry cv 'Aurora'.

Materials and methods

Plant material

1) F_1 hybrids were derived from crossing a colchicine-doubled tetraploid PF raspberry (R′R′R′R′, 2n = 4x = 28) with aneuoctaploid blackberry (BBBBBBBB, 2n = 8x = 58), allohexaploid hybridberry (RRBBBB, 2n = 6x = 42) and tetraploid blackberry (BBBB, 2n = 4x = 28) to give allohexaploid (R′R′BBBB, 2n = 6x = 42), allopentaploid (R′R′RBB, 2n = 5x = 35) and allotetraploid (R′R′BB, 2n = 4x = 28) hybrid-berries, respectively.
2) The allotetraploid hybrid-berries (R′R′BB) were backcrossed with tetraploid raspberry (R′R′R′R′) to give a plant with the genomic designation R′R′R′B (2n = 4x = 28).
3) The aneuoctaploid blackberry cv 'Aurora' (2n = 58), whose parentage includes allohexaploid raspberry × blackberry hybridberries, is derived from the progeny of the cross cv 'Zielinski' (BBBBBBBBBBBB) × cv 'Logan' (BBBBRR) crossed with the progeny of the cross cv 'Logan' (BBBBRR) × cv 'Austin Thornless' (BBBBBBBB).

Chromosome preparations

Root tips from pot-grown plants were collected in the morning and pretreated with a saturated solution of Gammexane® (hexachlorocyclohexane, Sigma) in water for 4 h and fixed in 3 : 1 absolute alcohol : glacial acetic acid for 24 h. Chromosome squashes were prepared as described by Schwarzacher and Leitch (1994) by enzyme digestion (0.2% (w/v) cellulase R10, 0.2% (w/v) pectolyase Y23 and 0.2% (w/v) driselase in 0.004 *M* citric acid, 0.0006 *M* tri-sodium citrate buffer) at 37°C for 28 min. The root caps of digested roots were removed gently with a needle and the meristem transferred onto a glass slide. The meristematic cells were macerated in a drop of 60% acetic acid and squashed gently under a coverslip which was then removed following liquid nitrogen freezing.

Genomic DNA extraction and labelling

Genomic DNA was extracted from young leaves of diploid raspberry (maternal selection 6330/28) and tetraploid blackberry (paternal selection 3947/1) following the method of Dellaporta et al. (1983). Genomic DNA was sheared to a mean length of approximately 12 kb with a syringe needle and labelled by nick translation with digoxigenin-11-dUTP (Boehringer Mannheim, Leitch et al. 1994) or biotin-14-dUTP (Sigma) following the manufacturer's instructions (BRL Life Technologies). Blocking DNA was sheared by boiling for 20 min.

rDNA probe

The probe pTa 71 was isolated from wheat (*Triticum aestivum*) and contains the 18S-5.8S-25S rRNA gene subunits and the non-transcribed spacer sequences in a 9-kb *Eco*RI fragment (Gerlach and Bedbrook 1979) recloned into pUC19 and provided by R.B. Flavell and M. O'Dell (John Innes Centre, Norwich). The entire plasmid was labelled with digoxigenin-11-dUTP by nick translation.

In situ hybridization

In situ hybridization was carried out as described in Leitch et al. (1994) with modifications. Slides were denatured in 70% formamide in 2 × SSC at 70°C for 2 min. The hybridization mixture contained 8 μg ml^{-1} digoxigenin or biotin-labelled raspberry DNA, 80–240 μg ml^{-1} blackberry blocking DNA, 4 μg ml^{-1} digoxigenin-labelled pTa 71, 50% (v/v) formamide, 10% (w/v) dextran sulphate and 0.1% (w/v) sodium dodecyl sulphate in 2 × SSC (0.3 *M* sodium chloride, 0.03 *M* sodium citrate). After overnight hybridization, the slides were washed in 20% (v/v) formamide in 0.1 × SSC at 42°C at an estimated hybridization stringency of 80–85%. Sites of probe hybridization were detected using 20 μg ml^{-1} fluorescein-conjugated anti-digoxigenin IgG (Boehringer Mannheim) and 5 μg ml^{-1} Cy3-conjugated avidin (Amersham) in 4 × SSC containing 0.2% (v/v) Tween 20 and 5% (w/v) bovine serum albumin. Chromosomes were counterstained with 2 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) in 4 × SSC, mounted in Vectashield (Vector Laboratories) medium, examined using a Leitz Aristoplan epifluorescent microscope and photographed using Fujicolor 400 colour film. Photographs were scanned and adjusted using Adobe photoshop. All images were treated for colour contrast and brightness uniformly.

Results

GISH analysis of allopolyploid hybrid-berry plant

DAPI staining of the chromosomes of blackberry, raspberry and hybrid-berry at metaphase after in situ

hybridization revealed small, metacentric and submetacentric chromosomes (e.g. Fig. 1A). The allopentaploid (R′R′RBB) shown in Fig. 1A,D was unusual in also having 1 near-telocentric chromosome (arrowed).

Results of GISH analysis on the allopentaploid (R′R′RBB) using digoxigenin-labelled total genomic raspberry DNA (R′R′) as a probe in conjunction with 30 × excess unlabelled blocking DNA from blackberry (BBBB) are shown in Fig. 1B–D. In 20 metaphases, 22 of the 35 chromosomes were labelled (Fig. 1B–C). The probe hybridized to the centromeric regions of 21 chromosomes, and 3 were also labelled along the whole short arm (centromere to telomere). The single near-telocentric chromosome had a signal at the telomeric end of the short arm.

A karyotype of the allopentaploid was prepared (Fig. 1D). The 21 chromosomes with clear labelling at their centromeres were considered to be of raspberry origin and arranged into groups of 3 based on similar morphology. The first group are metacentric and larger than the rest, and these are probably homologous. The last group with short arms entirely labelled are also considered homologous. Of the 13 unlabelled chromosomes 12 were considered to be of blackberry origin and were grouped by size and centromere position. The chromosome labelled at the telomere was the only near-telocentric chromosome present and so did not have an associated homologue. This was paired with a blackberry chromosome. At late prophase (Fig. 1F) the chromosomes of the allopentaploid (R′R′RBB) showed strong DAPI-positive centromeric regions; those of raspberry origin were labelled by GISH (Fig. 1G). DAPI-positive centromeric regions were also seen in late interphase/early prophase in the allotetraploid (R′R′R′B, Fig. 1H) with 21 chromosomes labelled with GISH, corresponding to the number of raspberry chromosomes (Fig. 1I).

GISH on the allotetraploid hybrid-berry (R′R′BB) labelled the centromeric regions of 14 chromosomes; 2 were also labelled along the entire short arm (Fig. 1E).

Characterization of the blackberry cv 'Aurora'

The aneuoctaploid blackberry cv 'Aurora' has a complex parentage (see Materials and methods) which includes crosses with raspberry. It has a chromosome number of 2n = 58 (Thompson et al. 1995, Fig. 1J) rather than 56 as might be expected from the simple multiplication of the basic chromosome number of 7.

GISH using total genomic DNA of raspberry in the presence of 10 × excess unlabelled blocking DNA from blackberry revealed 11–12 sites of hybridization. One chromosome showed a signal at both telomeric regions (Fig. 1J, arrow), 3 chromosomes labelled like raspberry chromosomes with a strong signal at or around the centromere (Fig. 1J, M arrowhead) and the remaining sites were small and terminal. The chromosome with the raspberry probe label at both telomeric regions was identified in several but not all metaphases. Double-labelling experiments in which total biotin-labelled raspberry DNA was hybridized to the chromosomes in the presence of blocking DNA from blackberry and digoxigenin-labelled pTa 71 for rDNA showed that cv 'Aurora' has six rDNA sites (Fig. 1L), all of which co-localized with the signal derived from the genomic probe, as can be seen by comparing Fig. 1K and 1L and in the overlay Fig. 1M.

rDNA localization

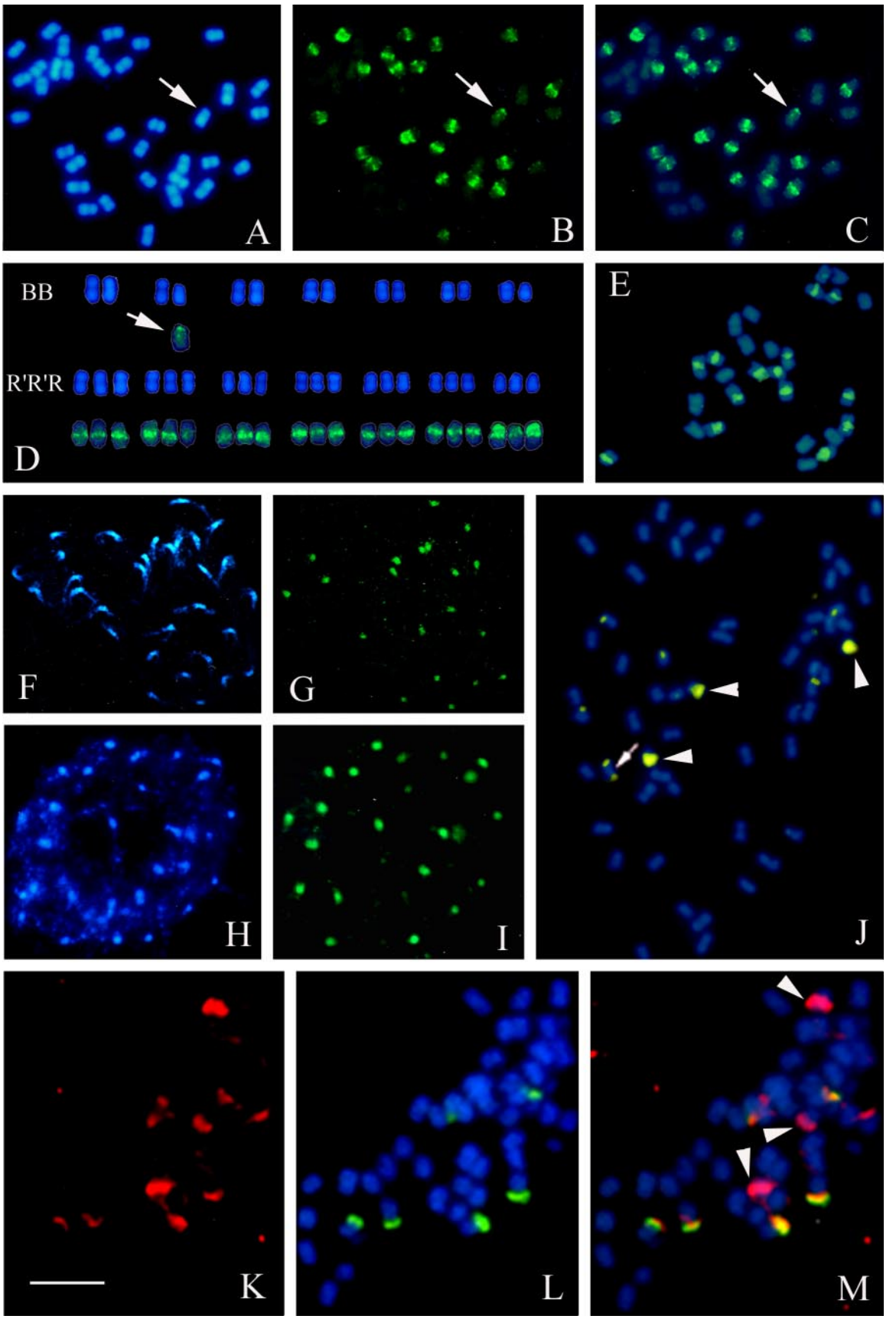
In diploid raspberry (2n = 2x = 14, R′R′), there were two rDNA sites (Fig. 2A). In all of the metaphases observed (> 20), one site appeared larger than the other. In the colchicine-doubled tetraploid raspberry (2n = 4x = 28), there were four rDNA sites, and one appeared larger than the rest (Fig. 2B). The same result was observed in the allotetraploid (R′R′BB), which had four rDNA sites, one markedly bigger than the rest (Fig. 2D). The allohexaploid (R′R′BBBB) had six rDNA sites, and again one locus appeared larger (Fig. 2E). At interphase (Fig. 2C) and prophase (Fig. 1H) there was typically one nucleous irrespective of ploidy or genomic composition. The nucleolus had an associated rDNA signal which appeared as bright foci both inside and outside the nucleolus and as diffuse strings of fluorescent dots within the nucleolus (Fig. 2C).

Discussion

Genome structure

Members of the genus *Rubus* have small genomes (1C = 0.3 pg, Bennett et al. 1997) and a basic chromosome number of x = 7. One chromosome of the basic set carries a 25S-5.8S-18S rDNA locus on the short arm (Fig. 2A,B), as was previously suggested from an analysis of secondary constrictions following standard cytological preparations (Pool et al. 1981). Thus, there are no additional, silent loci as occurs in other species (e.g. *Hordeum vulgare*, Leitch and Heslop-Harrison 1993). Similarly, the basic genome of *Rosa* (Rosaceae, n = x = 7) also has a single rDNA locus (Ma et al. 1997). Interestingly, rDNA loci were of different sizes in all the plants examined. Metaphases typically showed one site which was particularly large. It is interesting to speculate that this site was the most active at the preceding interphase. Differential expression of loci is a well-known phenomenon, especially in allopolyploids (e.g. the Gramineae, Flavell 1986; Leitch et al. 1992) but is less well established in autopolyploids and diploids.

GISH labelled the centromeric region of all raspberry chromosomes, coinciding with a region of strong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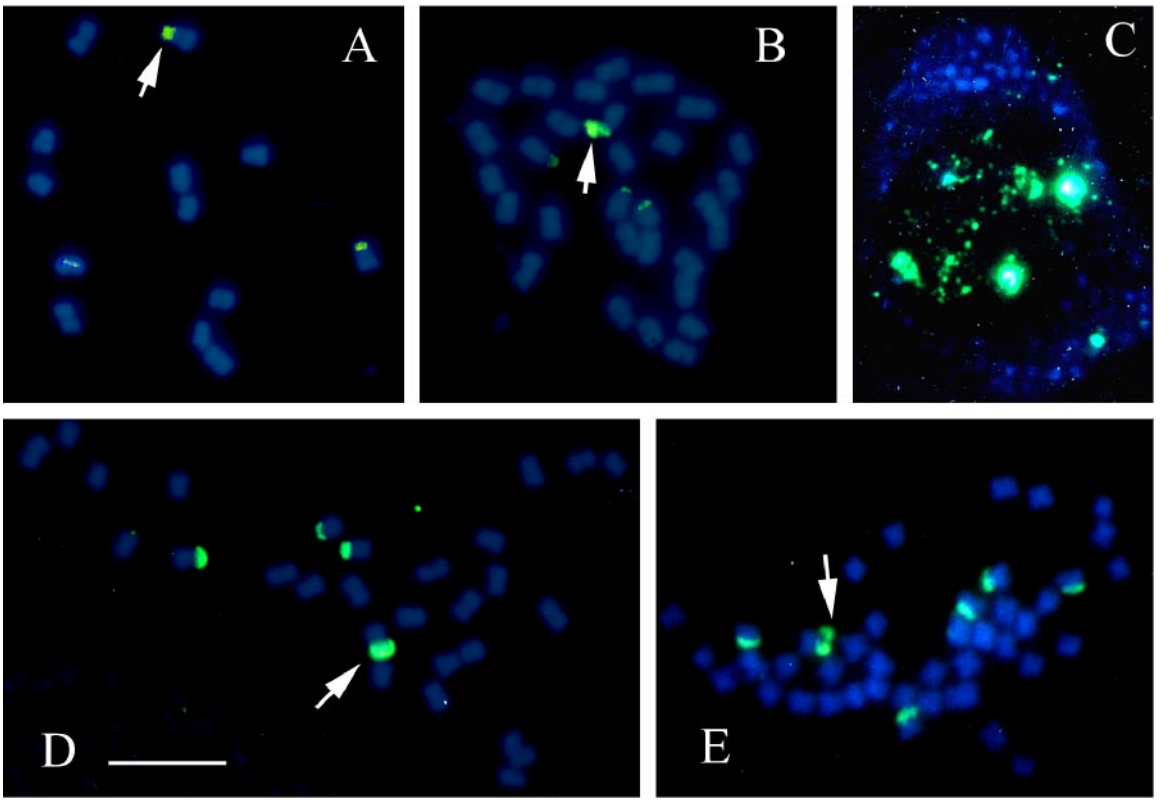

Fig. 2A–E FISH to localize rDNA in diploid and polyploid *Rubus*; sites of probe hybridisation have *yellow/green* fluorescence and are electronically superimposed onto the DAPI image for DNA (*blue* fluorescence). **A** Diploid raspberry (R′R′); two rDNA signals. **B** Colchicine-doubled raspberry (R′R′R′R′); four rDNA signals. **C** Interphase nucleus of allotetraploid hybrid-berry (R′R′R′B) with condensed rDNA (*bright* signal foci) and decondensed rDNA (*diffuse* signal) in the nucleolus. **D** Allotetraploid (R′R′BB); four rDNA signals. **E** Allohexaploid (R′R′BBBB); six rDNA signals. Note metaphase in **A**, **B**, **D** and **E** all have one rDNA site that is larger (*arrow*). *Scale bar*: 5 μm

Fig. 1 A–D Allopentaploid (R′R′RBB) metaphase chromosomes: *arrows* point to the near telocentric chromosome. **A** DAPI staining (*blue* fluorescence). **B** GISH using total raspberry DNA as a probe. Sites of probe hybridization have *yellow/green* fluorescence. **C** Electronic overlay of images in **A** and **B**. **D** Karyotype of metaphase showing DAPI-stained chromosomes (*blue* fluorescence – *top row*), *below*, the chromosomes following GISH (*bottom row*). Chromosomes are organized into their respective genomes (blackberry BB and raspberry R′R′R). **E** Allotetraploid (R′R′BB) labelled by GISH with raspberry probe (*yellow/green*) superimposed onto the DAPI image (*blue*); incomplete metaphase. **F**, **G** Partial late prophase of allopentaploid (R′R′RBB) showing DAPI-positive centromeric regions (**F**), some of which (**G**) are labelled by the raspberry genomic probe. **H**, **I** Early prophase of allotetraploid (R′R′R′B) showing DAPI-positive centromeric regions (**H**) and a central DAPI-negative nucleolus (**I**) GISH signal using the raspberry genomic probes labelling the 21 raspberry centromeric regions. **J** Aneuoctaploid blackberry cv 'Aurora' showing 58 DAPI-stained chromosomes probed with raspberry total genomic DNA. GISH and DAPI signals electronically overlaid. Note three large signals are of raspberry origin (*arrowheads*); 1 chromosome has signal at both terminal regions (*arrow*) **K–M** Blackberry cv 'Aurora' chromosomes labelled with biotinylated-raspberry DNA (*red* fluorescence) (**K**). **L** digoxigenin-labelled pTa 71 for rDNA (*yellow* fluorescence) overlaid electronically with DAPI fluorescence for chromosomes. **M** Electronic overlay of images **K** and **L**. There are six rDNA loci, all of which co-localize with the genomic raspberry probe. Three chromosomes (*arrowheads*) are strongly labelled. At least two further sites are labelled by the raspberry probe only. *Scale bar*: 10 μm (*A–C*, *E–J*); 7.5 μm (**D**); 5 μm (**K–M**)

DAPI staining. The chromosome arm which carries rDNA was also labelled by GISH. Similar results were obtained when GISH was used to identify chromosomes in other plants with small genomes e.g. *Brassica* (Fahleson et al. 1997). The centromeric region appeared condensed at prophase and interphase, and the heterochromatin occurred as foci across the nucleus. It is not absolutely certain what sequences are labelled by GISH, but major components of the signal are likely to be genome-specific, repetitive sequences (Jiang and Gill 1994; Heslop-Harrison and Schwarzacher 1996) including dispersed and tandem-repeated sequences. Because repeat sequences are the most likely components of the centromeric region of the chromosomes, the genic sequences are likely to be telomeric in location as occurs in many other organisms. Labelling these regions with cloned probes will be useful for identifying individual *Rubus* chromosomes as is now possible for other plants

with small chromosomes where chromosome banding and chromosome morphology is inadequate for chromosome identification (Jiang and Gill 1994; Jiang et al. 1995).

Breeding

The parentage of *Rubus* breeding material is often highly complex, and segregation at meiosis of plants with uneven genome dosages may give rise to gametes with variable chromosome numbers. We used FISH and GISH to determine the presence of raspberry and blackberry chromosomes and the occurrence of any translocations between genomes in blackberry × raspberry hybrid material.

The allopentaploid in Fig. 1A–D is derived from a cross between an autotetraploid (R′R′R′R′) and an allohexaploid (RRBBBB). Regular meiosis in the parental plants, in which genomes pair homologously, would give rise to gametes R′R′ and BBR, respectively, and an F_1 plant that is R′R′RBB. GISH revealed 21 probably complete raspberry chromosomes. In addition, there was a near-telocentric chromosome that had terminal raspberry chromatin. This chromosome may have arisen either by homoeologous paring and exchange between chromosomes of the B and R genomes in the allohexaploid parent or by a translocation event not associated with regular meiosis. Both possibilities would result in chromatin exchange between the B and R genomes. In other man-made allopolyploids (e.g. in the Graminae) such intergenomic translocations can become fixed (Jiang and Gill 1994; Heslop-Harrison and Schwarzacher 1996).

The blackberry cv 'Aurora' is aneuoctaploid ($2n = 8x = 58$) and, assuming regular bivalent formation and segregation in the parents of cv 'Aurora', we would expect seven complete blackberry genomes and 9 raspberry chromosomes. We observed: (1) 3 probably complete raspberry chromosomes: (2) 1 chromosome with two terminal raspberry signals; (3) at least 1 additional chromosome carrying terminal GISH signal which does not co-localize with rDNA (Fig. 1K–M) and (4) six rDNA loci. If there were seven blackberry genomes in the parents of cv 'Aurora', we would expect at least seven rDNA loci (one per B genome). The fact that we observed six rDNA loci suggests a loss of a locus either through the elimination of a B-genome chromosome or via a chromosomal translocation. The chromosome with raspberry signals at both telomeres is not seen in the raspberry genome, suggesting either a translocation or a centric inversion of a raspberry chromosome. There are at least 4 fewer raspberry chromosomes than expected with concomitant extra blackberry chromosomes. The occurrence of blackberry/raspberry recombinant chromosomes in the allopentaploid, and possibly in cv 'Aurora', shows that homoeologous pairing must be occurring. This is presumably disrupting the segregation of the blackberry genomes which is giving rise to the unexpected blackberry dosage in cv 'Aurora'.

The absence of the GISH signal over the entire length of raspberry chromosomes will limit the number of raspberry-blackberry translocations detected to those associated with the centromeric region. The subterminal and terminal regions of the chromosomes, which may be gene-rich, did not appear to label in the majority of the chromosomes. Nevertheless, the small extra terminal GISH signal detected in the allopentaploid (Fig. 1D) and the detection of translocated raspberry chromosomes in the aneuoctaploid blackberry cv 'Aurora' (Fig. 1J–M) demonstrates that there is potential to detect chromosome rearrangements and translocations in *Rubus*. There is now a need to find sequences that may be used to label the entire genome so that all translocations and rearrangements can be detected.

The use of GISH confirmed the expected genome dosages of the allotetraploid (R′R′BB), allopentaploid (R′R′RBB) and allohexaploid (R′R′BBBB) hybridberries and demonstrated the raspberry contribution in blackberry cv 'Aurora'. In blackberry, the fruit is an aggregate of droplets that detach at an abscission zone at the base of the torus and the fruit is removed with the torus. In raspberry, the droplets are removed from the torus, leaving the torus attached to the plant. The allopentaploid is the only allopolyploid that has a raspberry-like fruit detachment, and only this plant has more raspberry genomes than blackberry genomes. Perhaps a proportional gene dosage contribution from the two genomes is important for the expression of this character. In addition, a high proportion of allopentaploid (R′R′RBB) plants expressed the primocane fruiting characteristic carried by the R′ genome. Interestingly, allotetraploid (R′R′BB) and allohexaploid (R′R′BBBB) plants which also carry two R′ genomes more rarely, or never, showed this character. Therefore, it must be assumed that there is an interaction of the summer-fruiting genome (R) with the two primocane fruiting genomes (R′) to increase the levels of primocane fruiting expression in the allopentaploids (R′R′RBB).

Mapping of cloned marker sequences has been performed successfully in several major crop species including those with small genomes (Jiang et al. 1995; Shi et al. 1996; Fukui et al. 1997). Recent advances in genome mapping, including BAC-FISH, and the identification of many genes or gene clusters of agronomic importance will facilitate the development of cytogenetic maps (cf. Jiang and Gill 1994) which can be applied to *Rubus* genomes. This may enable precise gene targeting in future breeding experiments.

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