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R. J. Snowdon · W. Köhler · W. Friedt · A. Köhler

Genomic in situ hybridization in *Brassica* amphidiploids and interspecific hybrids

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Abstract Genomic in situ hybridization (GISH) methods were used to detect different genome components within *Brassica* amphidiploid species and to identify donor chromatin in hybrids between *Brassica napus* and *Raphanus sativus*. In *Brassica juncea* and *Brassica carinata* the respective diploid donor genomes could be reliably distinguished by GISH, as could all R-genome chromosomes in the intergeneric hybrids. The A- and C-genome components in *B. napus* could not be clearly distinguished from one another using GISH, confirming the considerable homoeology between these genomes. GISH methods will be extremely beneficial for monitoring chromatin transfer and introgression in interspecific *Brassica* hybrids.

Key words *Brassica* · *Raphanus* · Interspecific hybrids · Genomic in situ hybridization · GISH

Introduction

Introgression into crop plants of alien chromosomes, or chromosome segments, containing useful genes is a valuable method for crop improvement. Reliable monitoring of alien chromatin during introgression is critical for successful transfer (Jiang and Gill 1994). Genomic in situ hybridization (GISH) is an efficient and accurate technique for the determination of the levels and incorporation positions of alien chromatin, and has been applied to numerous interspecific and intergenomic plant hybrids in recent years, including wheat-barley hybrid lines (e.g. Mukai and Gill 1991), grass hybrids (e.g. Schwarzacher et al. 1989; Leitch et al. 1990), and wheat-alien addition (e.g. Chen et al. 1995) and translocation (e.g. Friebe et al. 1995) lines.

Hybrids between *Brassica napus* and related species can be relatively easily produced and are an ideal method for introducing pest and disease resistance genes, for example from the *Brassica nigra* B-genome (e.g. Chevre et al. 1996; Struss et al. 1996) or the R-genome of *Raphanus* (e.g. Thierfelder and Friedt 1995). In the present study we have developed methods for fluorescent GISH which enable the distinction of all *Brassica* diploid genome components apart from the highly similar A and C genomes. In addition, R-genome chromosomes were able to be detected in hybrids between *Raphanus sativus* and *B. napus*.

Fluorescence in situ hybridization (FISH) was first applied to *Brassica* by Maluszynska and Heslop-Harrison (1993), who determined the numbers of rDNA loci in diploid and amphidiploid species. Further *Brassica* repetitive sequences were physically characterized by Harrison and Heslop-Harrison (1995). Elsewhere we have described characterization and localization of rDNA loci in the *Brassica* A and C genomes (Snowdon et al. 1997), but few further physical mapping studies in *Brassica* have been published, probably due to the difficulties associated with generating suitable cytological preparations in these species. The small size of the chromosomes in *Brassica* and related species is particularly problematic for the identification of alien chromosomes in *Brassica* hybrids, and translocations cannot be reliably identified

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R. J. Snowdon (✉) · W. Köhler
Institute für Pflanzenbau und Pflanzenzüchtung II,
Justus-Liebig-Universität, Ludwigstrasse 27, D-35390 Giessen, Germany
Fax: + 49 641-9937549
E-mail: rod.snowdon@agrar.uni-giessen.de

W. Friedt
Institute für Pflanzenbau und Pflanzenzüchtung I,
Justus-Liebig-Universität, Ludwigstrasse 23, D-35390 Giessen, Germany

A. Köhler
Institut für Humangenetik, Schlangenzahl 14, D-35392 Giessen, Germany

by conventional karyotyping methods. In conjunction with techniques for improved cytological preparations, GISH methods will aid considerably in the monitoring of alien chromatin transfer and introgression in *Brassica* breeding programs using interspecific hybridization.

Materials and methods

Plant material

The plants used for investigation of the *Brassica* amphidiploid genomes were *B. juncea* L. (AABB; 2n = 36; acc. TM18-8-Hk7A3), *B. napus* L. (AACC; 2n = 38; spring oilseed rape cv *Callypso*) and *B. carinata* L. (BBCC; 2n = 34; acc. 21164-Hk6B8). These are the three amphidiploids derived from spontaneous hybrids of the respective *Brassica* diploid species (U 1935).

Detection of R-genome chromatin was performed in intergeneric crosses between spring oilseed rape (cv *Drakkar*) and nematode-resistant genotypes of oil-radish (*Raphanus sativus* ssp. *oleiferus*; cv *Fortissimo*). ACR-genome F_1 amphihaploids generated using ovule culture (embryo rescue) produced three lines exhibiting very high nematode resistance (Thierfelder and Friedt 1995). Allohexaploid (AACCRR) plants were produced by colchicine chromosome doubling of the resistant F_1 amphihaploids, as described by Thierfelder (1994), and backcross lines were generated by crossing with the oilseed rape cultivar *Lisandra* (Pan and Friedt 1996). The F_1 amphihaploid 2062 and the nematode-resistant BC_1 individual 2062/16 were tested to determine the usefulness of GISH in monitoring R-genome chromatin-introgression in the breeding program.

For GISH probes, genomic DNA was extracted from *B. rapa* L. (Chinese cabbage cv Hong Kong), *B. nigra* L. (accession sf224-1A94), *B. oleracea* L. (white cabbage cv Braunschweiger), *B. napus* ssp. *oleifera* (spring oilseed rape cv Drakkar) and *R. sativus* ssp. *oleiferus* (oil-radish cv Fortissimo) using the CTAB-extraction method of Doyle and Doyle (1990). *B. rapa* is diploid for the *Brassica* A genome (AA; 2n = 20), *B. nigra* represents the B genome (BB; 2n = 16), and *B. oleracea* the C genome (CC; 2n = 18). *R. sativus* is an R-genome diploid (RR; 2n = 18).

GISH probes

Genomic DNA from the respective diploid species was labelled with digoxigenin by nick translation until the lengths of probe fragments, determined by agarose-gel electrophoresis, averaged approximately 500 bp. Labelled probes were re-suspended at a concentration of 250 ng/ml in a hybridization solution of 2 × SSC, 50% formamide and 10% dextran sulphate. A 50-fold excess of unlabelled blocking DNA (from the appropriate unlabelled diploid genomes, or from *B. napus* for the hybridizations with labelled R-genome DNA), produced by autoclaving genomic DNA 7 min at 1 bar to generate fragments of approximately 300–500 bp in length, was added to each probe solution. Probes were denatured for 10 min at 75°C and stored at −20°C until use.

Cytological preparations and in situ hybridization

Cytological preparations from *Brassica* species were produced from the root tips of young seedlings. For the *B. napus* × *R. sativus* hybrids, however, only adult plants were available and the best preparations were obtained from the tips of young roots generated from cuttings. In all cases, plant growth was synchronised by overnight refrigeration followed by a further 24 h growth at 25°C. Metaphases were then accumulated by incubating whole seedlings, or excised roots, for 2 h with vigorous shaking in 2 mM of 8-hydroxyquinoline. Material was fixed in several changes of Farmer's solution (3 : 1 ethanol : acetic acid) and stored at −20°C. After 90-min digestion in a cellulase-pectinase mix (Schwarzacher et al. 1994), root tips were subjected to a 25-min hypotonic treatment in 75 mM KCl, followed by 20-min washing in several changes of 60% acetic acid to clear the cytoplasm. Protoplasts were dispersed in Farmer's solution and chromosome spreads were made on chilled slides. Slides were checked by phase-contrast microscopy and preparations with an inadequate metaphase number or unacceptable background were discarded. Suitable preparations were treated with RNAase, fixed with formaldehyde and denatured following methods described previously (Snowdon et al. 1997). Probes were pre-annealed prior to hybridization, based on the procedure of Anamthawat-Jónsson and Reader (1995), by incubation for 5 min at 32°C. For GISH with A- and C-genome probes in *B. napus*, longer pre-annealing times were also investigated. In situ hybridization followed the method of Schwarzacher et al. (1994), with a stringent wash at 42°C for 10 min in 2 × SSC, 50% formamide.

Hybridization signals were detected using rhodamine-conjugated anti-digoxigenin with one round of routine signal amplification. Chromosomes were lightly counterstained for 1 min in 1 μg/ml of DAPI. Slides were mounted in antifade and examined using a Zeiss Axiophot microscope equipped with the appropriate filter sets for DAPI and rhodamine/DAPI. At least ten metaphases were examined for each hybridization. Photographs were taken on Kodak Ektachrome 400 colour slide film.

Results

High numbers of good metaphases and prometaphases were obtained in most cases, although accumulation of metaphases was much more reliable from seedlings than from cuttings of adult plants. Examples of in situ hybridizations with the different genomic probes in the respective *Brassica* amphidiploids and *B. napus* × *R. sativus* hybrids are shown in Fig. 1.

On metaphases and prometaphases, GISH distinguished all chromosomes of the A and B genomes in *B. juncea*, and the B and C genomes in *B. carinata*, respectively (Figs. 1a–d). In *B. napus*, excessive cross-genome hybridization prevented the reliable distinction of A- and C-genome chromosomes, with the respective A- and C-genomic probes hybridizing to almost all *B. napus* chromosomes under the standard hybridization conditions. Despite pre-annealing to block homoeologous sequences, labelled C-genome probes still hybridized to a number of A-genome chromosomes (Fig. 1 e). In hybridizations with labelled A-genome DNA (Fig. 1 f), only two to four *B. napus* chromosome pairs hybridized strongly; however, more than 20 further chromosomes showed significant hybridization signals. Longer (up to 25 min) pre-annealing reduced the strength of all but the strongest signals (e.g. Fig. 1 g), meaning that a clear discrimination between the genomes was still impossible. A distinction between A- and C-genome chromosomes according to differential hybridization strengths was not possible.

The *B. napus* × *R. sativus* amphihaploid hybrid 2062 contains 28 chromosomes, corresponding to the sum of

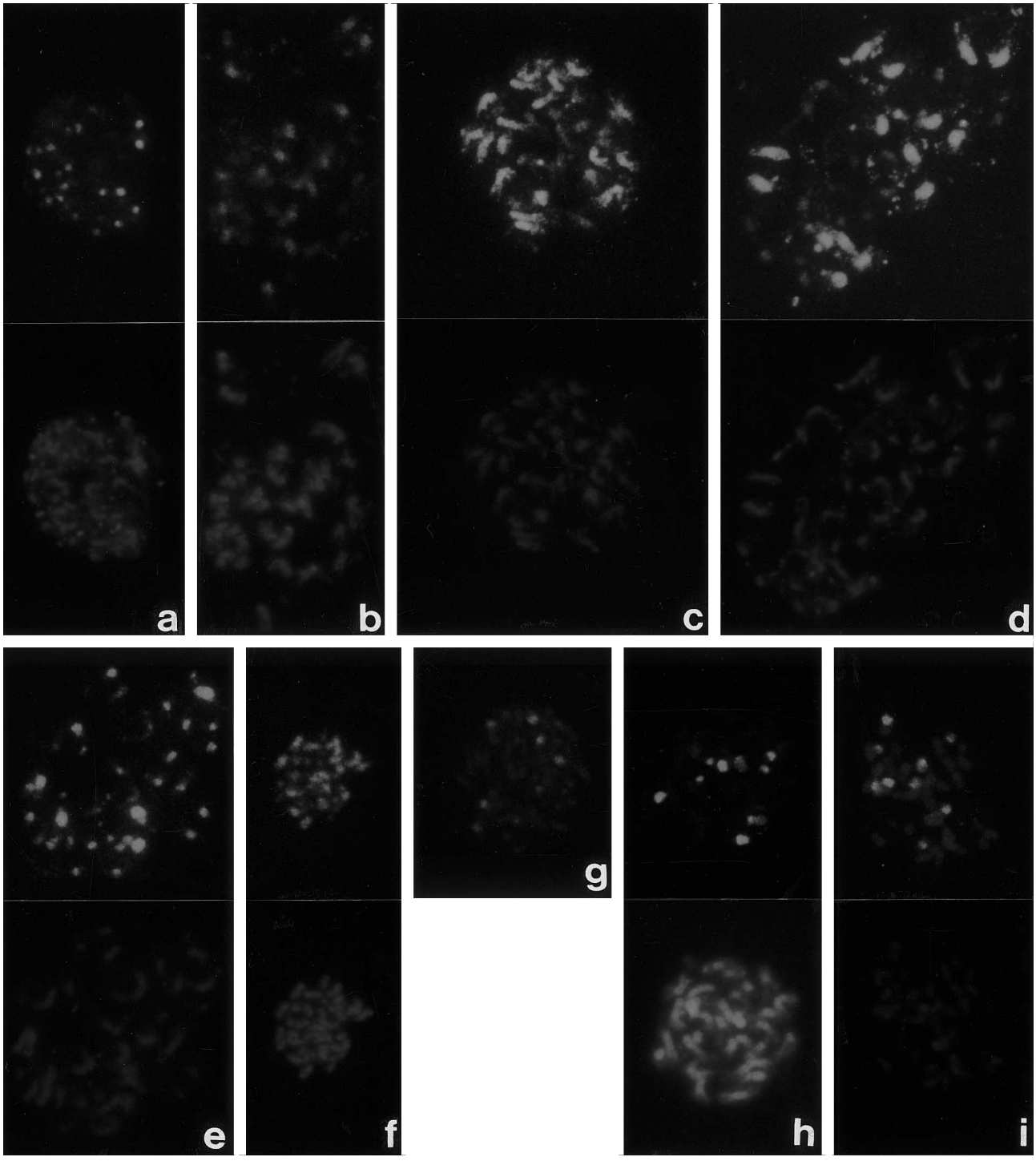

Fig. 1a–i Genomic in situ hybridization of diploid genome probes in *Brassica* amphidiploids and of *Raphanus* DNA in *B. napus* × *R. sativus* hybrids. Hybridization sites are detected with rhodamine (above) and chromatin is stained with DAPI (below). **a, b** Detection of A genome (**a**) and B genome (**b**) chromosomes in a *B. juncea* metaphase and prometaphase, respectively. (**c, d**) Identification of C-genome (**c**) and B-genome (**d**) chromosomes in prometaphases of *B. carinata*. **e, f** Hybridization of the A-genome (**e**) and C-genome (**f**) probes to a *B. napus* metaphase and prometaphase, respectively. (**g**) After extended pre-annealing, hybridization of the A-genome probe was blocked on almost all *B. napus* chromosomes. **h** Detection of the nine R-genome chromosomes in the amphihaploid *B. napus* × *R. sativus* F_1 hybrid 2062 (ACR; n = 28), and **i** nine *Raphanus* addition chromosomes in the BC_1 hybrid 2062/16 (AACCR; 2n = 47)

chromosomes from the haploid genomes of *B. napus* (AC; n = 19) and *R. sativus* (R; n = 9). All nine *Raphanus* chromosomes could be clearly distinguished by GISH with labelled *R. sativus* DNA (Fig. 1 h). The backcross individual 2062/16 has a chromosome number of 2n = 47, with 38 *B. napus* chromosomes showing no significant hybridization. GISH allowed the identification of nine *R. sativus* addition chromosomes (Fig. 1 i).

In all cases, hybridization signals were strongest in the centromeric regions, and signals at the telomeres were unexpectedly weak. Variation in signal intensity among chromosomes was also noted, with some consistently exhibiting reduced signals. This phenomenon was particularly noticeable for two *R. sativus* chromosomes which consistently showed less intense hybridization.

Optimization of blocking DNA concentrations and pre-annealing times improved the uniform hybridization of probes over the complete length of target chromosomes, but also resulted in minor cross-genomic hybridization. Cross-hybridization could be easily distinguished as such, however, because signals were comparatively very weak and often occurred not at centromeres but on chromosome satellites, where we have previously identified large rDNA loci (Snowdon et al. 1997).

Discussion

The results demonstrate that GISH can be effectively applied to detect donor genomes in *Brassica* hybrids. Aided by biotechnological advances that overcome incompatibility problems (e.g. Thierfelder 1994), interspecific hybridization is now an important technique for gene transfer into and among *Brassica* species. GISH methods for *Brassica* will provide an important tool to monitor the success of donor genome introgression.

The above techniques were applied to material from an ongoing breeding program aimed at the transfer of nematode-resistance genes from oil-radish to oilseed rape. Although this breeding program is still in its early stages, and the first backcross lines contain excessive amounts of the oil-radish genome, it was demonstrated that GISH enables effective monitoring of introgression of *Raphanus* chromatin in the hybrid offspring. In subsequent backcross lines, individuals exhibiting high levels of nematode resistance will be subjected to cytological and GISH analysis to select fertile resistant plants with minimal levels of donor chromatin. The methods described here can also be applied to other *Brassica* breeding programs involving the introgression of alien chromatin by wide crosses. In particular it was shown that the B genome of *B. nigra* can be readily distinguished from other *Brassica* genomes using GISH. Along with *Raphanus*, *B. nigra* is another common source of pest- and disease-resistance genes for *Brassica* breeding (e.g. Chevre et al. 1996; Struss et al. 1996).

The only instance where a distinction between genomes was not possible using GISH involved the two diploid genome components of *B. napus*. The extremely high homoeology between the A and C genomes concerned has been well documented using molecular markers (e.g. Parkin et al. 1995; Cheung et al. 1997). It is thus not surprising that GISH probes for *B. napus* show excessive intergenomic cross-hybridization between the A and C genomes. Without pre-annealing, all *B. napus* chromosomes were strongly labelled by both the A- and C-genome probes (data not shown). Pre-annealing resulted in the reduction of signals on chromosomes of both genomes, with only a few chromosomes in each case still showing strong hybridization. Longer pre-annealing blocked significant hybridization of A-genome DNA to all but two-to-four chromosome pairs, indicating that highly-repetitive sequences specific to the *Brassica* A genome seem to be present in only a small number of chromosomes. Further optimization of GISH and pre-annealing conditions for *B. napus* might offer additional insight into the evolutionary relationships and homoeology between its genome components, but alternative methods to GISH may be needed to distinguish between chromosomes from the A and C genomes. For example, Harrison and Heslop-Harrison (1995) isolated *Brassica* centromere sequences which were not present in the B genome, and an A-genome centromeric repeat not present in the C genome was described by Iwabuchi et al. (1991). The discovery of further repeat sequences specific to the A or C genomes might allow for the identification of some or all chromosomes from these genomes. Of particular use might be interspersed tandem repeat sequences that hybridize over the full length of chromosomes. We are investigating the possibility of using RAPD-PCR to generate genome-specific interspersed repeat probes for FISH.

One major problem in the development of GISH methods for *Brassica* was obtaining uniform hybridization over the full length of chromosomes. In early results, hybridization on chromosome arms was minimal or absent. This phenomenon could be caused by a higher intergenomic homoeology of repetitive sequences on chromosome arms than at the centromeres, with a consequent stronger blocking of these regions, or possibly by a concentration of repetitive sequences at centromeres. Another speculative explanation might be that chromosome arms contain predominantly AT-rich repetitive sequences, which can be preferentially degraded during heat denaturation. Telomeres often exhibited bright DAPI-bands, indicating heterochromatic blocks (Schwarzacher and Heslop-Harrison 1991), and the lack of significant GISH signals here was unexpected. A similar preferential hybridization of centromeres is often observed in *Brassica* GISH (J. Maluzynska, personal communication) and would

present difficulties in the identification of non-centromeric intergenomic translocations. Achieving uniform hybridization over the full length of chromosomes was thus a major focus of our methodological improvement, and both the balance of labelled probe to unlabelled blocking DNA, as well as the pre-hybridization treatment of chromosomes, appeared critical for hybridization over whole chromosomes. Over-denatured chromosomes showed reduced hybridization to chromosome arms and optimization of slide treatment and denaturing conditions was vital. Subsequently, concentrations of blocking DNA were reduced until signals were optimized without excessive intergenomic cross-hybridization.

Cross-hybridization, along with over-hybridization of repetitive sequences (particularly at centromeres), was further reduced by short pre-annealing of homoeologous repetitive probe sequences, based on the procedure of Anamthawat-Jónsson and Reader (1995). With the exception of the highly similar A and C genomes of *B. napus*, the combination of reduced blocking DNA and brief pre-annealing resulted in more uniform hybridization over the full length of target chromosomes and considerably reduced cross-genome hybridization. Minor cross-hybridization was often observed, but could normally be distinguished as such because signals were relatively weak and often occurred on chromosome satellites. *Brassica* NOR-satellites correspond to positions where we have identified large rDNA loci (Snowdon et al. 1997). Weak intergenomic cross-hybridization to rDNA loci is not unexpected, owing to the similarity between the genomes investigated and the fact that rDNA repeat sequences have an extremely high copy number in *Brassica* (Bennett and Smith 1991).

The ability to hybridize GISH probes uniformly over the whole length of chromosomes, without interfering cross-genomic hybridization, is critical for the identification of translocations in intergenomic hybrids. Ideally, a breeding program utilising interspecific hybridization should result in fertile hybrid lines carrying genes of agronomic interest but with minimal extra donor chromatin. The GISH methods which have been described here will allow for the screening of backcross progeny from interspecific *Brassica* hybrids, exhibiting the desired transferred trait, for valuable intergenomic translocations.

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