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Advances in plant chromosome identification and cytogenetic techniques

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Recent developments that improve our ability to distinguish slightly diverged genomes from each other, as well as to distinguish each of the nonhomologous chromosomes within a genome, add a new dimension to the study of plant genomics. Differences in repetitive sequences among different species have been used to develop multicolor fluorescent *in situ* hybridization techniques that can define the components of allopolyploids in detail and reveal introgression between species. Bacterial artificial chromosome probes and repetitive sequence arrays have been used to distinguish each of the nonhomologous somatic chromosomes within a species. Such karyotype analysis opens new avenues for the study of chromosomal variation and behavior, as well as for the localization of individual genes and transgenes to genomic position.

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Introduction

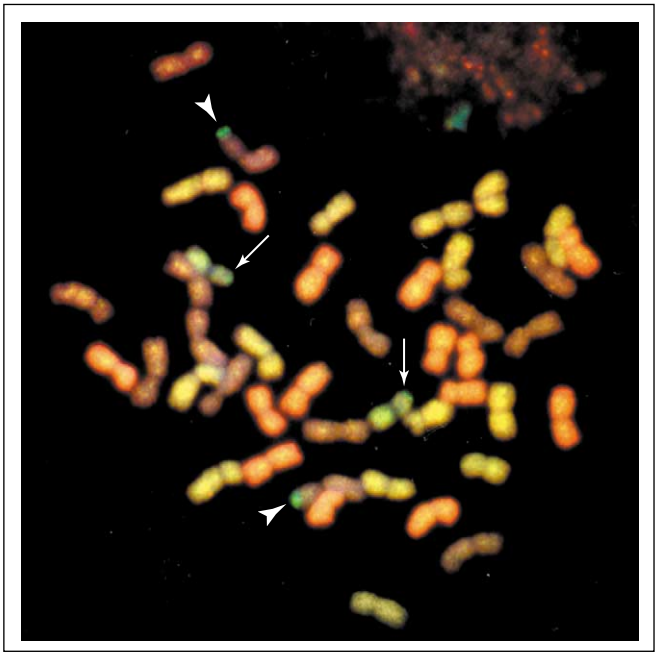
Chromosomal identification procedures allow investigators to use fluorescent signals that identify specific sequences, chromosomes, chromosomal segments or whole sets of chromosomes to gain a genome-wide view at a single glance. Such a perspective is valuable for many applications including a determination of the genome-wide distribution of repetitive sequences, visualization of genomic rearrangements in individual cells and analysis of chromosomal behavior. Cytogenetic techniques have become necessary components of studies of the organization of the genome and its association with chromatin. In this review, we summarize recent developments in techniques for distinguishing chromosomes from different genomes and for differentiating the nonhomologous components within a genome. We also discuss other related cytological tools.

Genomic *in situ* hybridization

The first procedure to use fluorescent labels to distinguish chromosomes in the plant kingdom involved the process of genomic *in situ* hybridization (GISH) [1]. This widely applied cytogenetic technique provides a direct visual method for distinguishing parental genomes and analyzing genome organization in interspecific hybrids, allopolyploid species and interspecific introgression lines [2]. In GISH, labeled total genomic DNA is used as a probe with unlabeled genomic DNA from another species as a blocking agent. Because the chromosomal sequences that are common to the two species contributing to the analyzed specimen are hybridized with unlabeled DNA, the labeled probe, especially the portion containing species-specific dispersed repetitive sequences (such as transposable elements), hybridizes to only one of the two sets of chromosomes.

Because of the allopolyploid nature of wheat and other members of the Triticeae tribe, their genomes have been extensively studied by GISH. This technique has a practical application in identifying alien chromatin introgression from different species as well as for studying chromosomal pairing and recombination between divergent genomes. Discriminating between two genomes of distant genera is easier than discriminating genomes within the same genus. Thus, the identification of the three closely related genomes in allohexaploid wheat (*Triticum aestivum* L.) is difficult. Recently, Han and colleagues [3••,4•] developed a modification of the multicolor GISH method to overcome this problem. Before this work, the identification of the A, B and D genomes of common wheat using GISH had been reported by Mukai *et al.* [5] and Sanchez-Moran *et al.* [6]. However, the methods used in these studies did not work consistently, and that used by Sanchez-Moran *et al.* [6] did not easily distinguish the A and D genomes. A new approach by Han *et al.* [3••] overcomes these problems because the results using this methodology can clearly discriminate the three genomes of wheat and of introgressed alien chromatin (Figure 1). Total genomic DNA of *Thinopyrum intermedium* and *Triticum urartu* was labeled with digoxigenin-11-dUTP, and total genomic DNA of *Aegilops tauschii* was labeled with biotin-16-dUTP by the nick-translation method. Total genomic DNA of

Figure 1



Multicolor GISH pattern of a wheat–*Thinopyrum intermedium* addition line. The A-genome chromosomes were detected as yellow fluorescence, the D-genome chromosomes were revealed by red fluorescence, and the B-genome chromosomes appeared as a brown color as a result of cross hybridization of different genomic probes. The alien chromosomes or fragments of *Th. intermedium* were revealed by green fluorescence. Arrowheads indicate one pair of wheat–*Th. intermedium* translocation chromosomes involving the wheat B genome and *Th. intermedium* chromosome segments (brown and green). Arrows indicate that a pair of *Th. intermedium* chromosomes has been added (green). (Photo by F Han.)

Aegilops speltoides was used for blocking. Detection of the biotinylated probe was accomplished with avidin-XRITC and digoxigenin using a fluorescent antibody enhancer set. The chromosomes were counterstained with DAPI. This multicolor GISH provides a powerful technique that could be generalized to determine the genomic constitution and variation in allopolyploids of other plant groups.

Karyotyping using FISH

Rather than distinguishing chromosomes from different genomes in hybrids or allopolyploids, karyotyping techniques are used to differentiate the nonhomologous chromosomes within a genome. Such procedures are important for detecting chromosomal aberrations, for defining which chromosomes are involved in cases of aneuploidy, for studies of chromosomal behavior and for the genomic localization of repetitive DNA sequence arrays, individual loci or transgene insertion sites.

The most commonly used probes for fluorescence *in situ* hybridization (FISH) karyotyping in plant species are the 5S and 25S rRNA genes, tandemly repeated sequences near telomeres, and centromere-specific repeats. A limited number of tandemly repeated sequences, which provide a large target for hybridization by a single probe, makes chromosome identification possible by FISH procedures in *Arabidopsis thaliana* [7], *Pinus* species [8] and Norway spruce [9]; however, in the latter two cases there are some ambiguities in the ability to distinguish different members of the karyotype. By combining these FISH landmarks with other chromosomal characters such as arm ratio and heterochromatic regions, precise karyotyping has been reported for the cytologically well characterized KYS inbred line of maize [10,11] and for selected lily species [12].

Considerable progress was made recently in the process of identifying unique DNA sequences that can be used for chromosomal identification in multicolor FISH procedures on plant chromosomes. Lysak and colleagues [13,14••] demonstrated the use of bacterial artificial chromosome (BAC) clones for chromosome painting of *Arabidopsis* species, namely, the separate labeling of chromosomes to visualize each in different colors. Because of its small genome size and paucity of repeated sequences, the chromosome arms of *Arabidopsis* were successfully painted with contiguous BACs of a total length of 2.6–14.4 Mb. The ability to paint chromosomes makes it possible to visualize individual chromosomal behavior throughout the cell cycle. In several plant species, individual BACs have also been used successfully as FISH probes to detect the chromosomal location of specific sequences [15–17]. In tomato [18], sorghum [19] and potato [20], BACs that are specific to a chromosome

arm were stably mapped using low Cot DNA suppression hybridization. This process blocks the nonspecific hybridization of the repetitive sequences by consuming repeated DNA sequences in the probe DNA by preannealing it with a low Cot fraction of highly repeated sequences or by saturating the repeated sequences on the chromosomes. Thus, only the sequences in the BAC that are not abundant in the target genome can hybridize to the target.

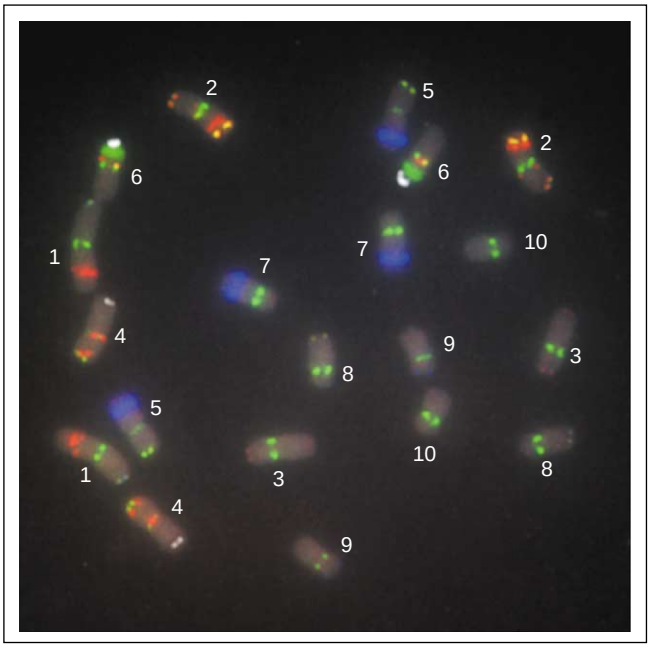
Screening BACs using FISH on wheat [21] and *Silene latifolia* [22] has been carried out for a different purpose. In this case, they were used to identify tandemly arrayed repeats that could be used for chromosome identification. Several BACs containing useful repetitive sequences that showed unique banding patterns on the chromosomes were isolated and could be used for karyotyping purposes.

Koumbaris and Bass [23•] used labeled *Sorghum* BACs containing markers common to chromosome 9 of maize and succeeded in labeling maize chromosomal segments that are homologous to selected *Sorghum* BACs in an effort to develop a combined physical-genetic map of maize. Their success is based on the higher gene density of the *Sorghum* BACs than maize BACs, and the divergence of the repetitive elements between the two species. Detection of maize BACs or cosmids on maize chromosomes by suppression hybridization is possible but the background levels are high [24,25] because of the presence of a high level of dispersed repetitive sequences in the genome.

Another approach to identifying new FISH probes for chromosome karyotyping is FISH screening of subtracted random PCR libraries, as demonstrated by Kato and colleagues [26••]. During the screening, a (TAG)n microsatellite and chromosome-specific subtelomeric sequences were recovered. These two repeat arrays show distinct patterns on several maize chromosomes. By simultaneously using nine probes, including the two newly identified (TAG)n microsatellite and chromosome-specific subtelomeric sequences, a multicolor FISH procedure identified each of the ten maize somatic chromosomes in all inbred lines and varieties tested to date ([26••]; Figure 2). This approach of increasing the number of repetitive array probes could be applied to other species to improve the technique's ability to distinguish the different nonhomologous chromosomes in a karyotype.

Conventionally labeled biotin or digoxigenin probes, that are detected by subsequent fluorochrome conjugated antibody or streptavidin application, have been used for multicolor FISH procedures [27]. However, for karyotyping purposes, in our experience, probes that directly incorporate fluorochrome-labeled nucleotides give more

Figure 2



Identification of the ten pairs of somatic chromosomes of maize inbred line B37 using nine fluorochrome-labeled DNA probes. A pair of each homologous chromosome of the karyotype is numbered. The nine probes were knob 180-bp repeat (revealed as blue), 5S rDNA (yellow); NOR on chromosome 6 short arm, CentC, and subtelomeric 4-12-1 (revealed as green), Cent4, microsatellite TAG repeat and pMTY9ER telomere associated sequence (revealed as red), and TR 1 knob repeat (white). Further description of the probes and the karyotyping procedure can be found in Kato *et al*. [26••]. (Photograph by A Kato.)

consistent signal presence on chromatin and improved signal to background ratios than do the antibody systems. A variety of DNA-labeling systems are available commercially; however, nick translation works well for karyotyping [26••].

Because the identification of chromosomes using FISH probes is not dependent on the extent of chromosome condensation or measuring arm ratios, individual chromosomes can be identified and studied at various stages in the cell cycle. For example, whole or partial chromosome paints in *Arabidopsis* can distinguish chromosomes at pachytene of meiosis and have been used to locate chromosomes in mitotic interphase when no chromatin condensation has occurred [14••,28]. Kato and colleagues [26••] successfully distinguished each chromosome in the maize complement at meiotic anaphase using the complete FISH probe cocktail described above.

Bass and colleagues [29] investigated maize chromosome meiotic behavior in an oat genetic background using a technique similar to GISH to examine homologous pairing, which revealed that synapsis is initiated at the late leptotene stage. The ability to locate chromosomal regions without dependence on chromosome shape has allowed studies on nuclear architecture in *Arabidopsis* during interphase [28] and meiotic processes [30].

Transgene detection

Chromosomal position and local chromatin structure are thought to have a profound effect on the level of gene expression. Variable transgene expression and silencing occur frequently in transgenic plants. In the past few years, there has been much interest in the correlation between transgene expression and cytogenetic position [31••]. In plants that have been genetically engineered by particle bombardment or *Agrobacterium*-mediated transformation, the integration mechanism does not appear to be sequence-dependent and presumably occurs via illegitimate recombination at double-strand breaks in the genomic DNA [32]. Thus, in *Arabidopsis*, studies of insertion sites of *Agrobacterium* tumor-inducing DNA (T-DNA) used for transformation [33], identified by the isolation of flanking sequences, suggest an unbiased distribution among the five chromosomes. However, reports of transgene detection by FISH in barley [34] and other cereal species indicate a tendency toward the localization of transgenes in distal (i.e. subtelomeric and telomeric) chromosomal regions. These results can be reconciled by comparing the distribution of T-DNA insertions sites with the distribution of genes in these species. The small *Arabidopsis* genome consists of 85% gene-rich regions that are distributed essentially randomly along the chromosomes, whereas in the cereals examined, the genes constitute only 10–20% of the genome and are located at more distal chromosomal sites. Sequence analysis of the DNA flanking the insertion site sequences in *Arabidopsis* [33] and rice [35] has confirmed the preferential recovery of T-DNA in gene-rich regions.

The structure of the transgene loci has also been analyzed by FISH on interphase nuclei, metaphase chromosomes and on extended DNA fibers [36–39]. Particle bombardment often generates very large, high-copy-number transgenic arrays that can extend for megabases. Interestingly, earlier studies showed that dispersed metaphase FISH signals come together at interphase [36]. By contrast, *Agrobacterium* transformation gives rise to lower transgene copy numbers, and is usually characterized by single discrete FISH signals that are difficult to detect. Improvements in the limits of FISH detection would facilitate the cytogenetic analysis of transgene integration and interaction.

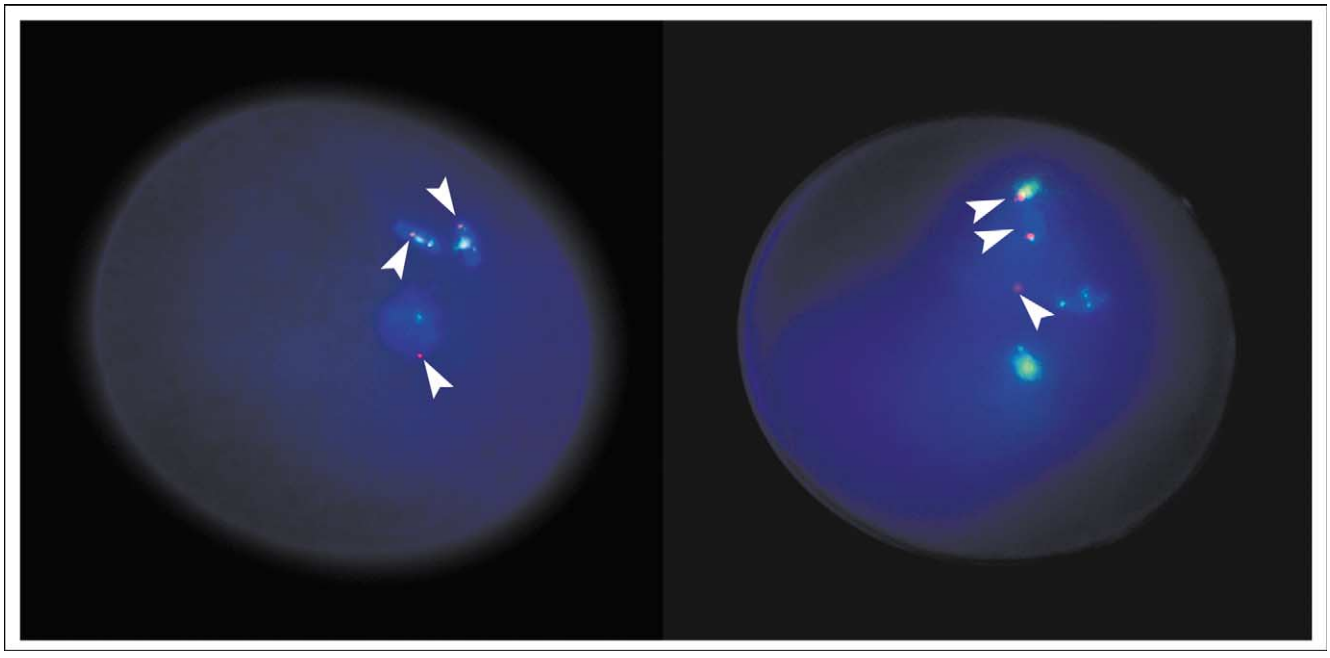
Fiber FISH, chromatin analysis and miscellaneous techniques

After the introduction of the fiber FISH technique (which applies fluorescently labeled probes onto naked DNA targets) to plant species [40], the procedure has been used to reveal the fine detail of DNA structure [41,42,43•] and to confirm the order of BACs in contigs assembled by other methods [44]. Undesirable DNA rearrangements can be generated during the creation or propagation of BAC libraries, especially in regions that contain highly repetitive sequences. Nagaki and colleagues [43•] compared the FISH probe hybridization signals of centromeric DNA elements on genomic fragments with the location on the corresponding BAC to confirm the lack of any major rearrangements.

The use of stretched chromatin, a procedure involving a gentle extension of the chromosome without removing the associated proteins, has become a useful tool with which to examine where various chromatin proteins interact with specific DNA sequences. In both maize and *Arabidopsis*, stretched chromatin was labeled with antibodies against the centromeric histone protein CENH3 and with centromeric DNA repeats as FISH probes [45••,46]. This technique allowed the arrangement of centromeric DNA elements and associated proteins to be revealed at high resolution.

Another recent study involved extended chromosomes and examined the detection of FISH signals on stretched somatic chromosomes [47••]. The procedure used flow-sorted barley and wheat mitotic chromosomes that were expanded to 10–100 times their original length. After *in situ* hybridization, the fine structure of the target sites on the chromosomes was revealed. This procedure provides better resolution of gene and repeated sequence arrangements on a chromosome while maintaining the information about the orientation relative to the centromere and telomere.

Figure 3



FISH on maize pollen. The red signal represents the supernumerary B-chromosome-specific signal in the sperm (arrows). The green signal identifies the heterochromatin knob 180-bp sequences, present mainly on the normal A chromosomes. The DNA of the vegetative nucleus and the two sperm cells were stained with DAPI (blue). **(a)** Pollen FISH of a maize pollen grain carrying a B chromosome derivative that underwent normal disjunction at both pollen mitoses to produce the large vegetative nucleus and the two small crescent shaped sperm cells. The B probe hybridizes to all three nuclei. **(b)** FISH on maize pollen carrying a normal B chromosome that shows nondisjunction at the second pollen mitosis so that one sperm has two B chromosomes and the other has none. The B chromosome signals (centromere and long arm tip) in the vegetative nucleus are weak because they are partially out of the focal plane of the two sperm cells. (Photograph by F Han.)

Last, the development of a FISH procedure for pollen grains has recently been reported [48–51]. This technique allowed the investigators to study the behavior of maize supernumerary B chromosomes in the pollen mitoses (Figure 3). The B chromosome contains specific sequence repeats that allow its visualization. This technique established that B chromosome nondisjunction occurs at a low level at the first pollen mitosis and at a much higher rate at the second pollen mitosis as predicted from genetic experiments.

Conclusions

The recent developments in cytogenetics described above will provide new tools for the analysis of plant genomes. These techniques allow the study of the fine details of chromosome structure and will permit sophisticated analyses of chromosomal behavior. As more genomes become sequenced, tools to study chromosomal organization and behavior will play a greater role in investigating the function of those genomes.

Acknowledgements

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