

Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research

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Abstract: Fluorescence in situ hybridization (FISH), which allows direct mapping of DNA sequences on chromosomes, has become the most important technique in plant molecular cytogenetics research. Repetitive DNA sequence can generate unique FISH patterns on individual chromosomes for karyotyping and phylogenetic analysis. FISH on meiotic pachytene chromosomes coupled with digital imaging systems has become an efficient method to develop physical maps in plant species. FISH on extended DNA fibers provides a high-resolution mapping approach to analyze large DNA molecules and to characterize large genomic loci. FISH-based physical mapping provides a valuable complementary approach in genome sequencing and map-based cloning research. We expect that FISH will continue to play an important role in relating DNA sequence information to chromosome biology. FISH coupled with immunoassays will be increasingly used to study features of chromatin at the cytological level that control expression and regulation of genes.

Key words: FISH, chromosome biology, genome research, molecular cytogenetics.

Résumé : L'hybridation in situ en fluorescence (FISH), laquelle permet de positionner directement une séquence d'ADN sur un chromosome, est devenue la technique la plus importante en cytogénétique moléculaire chez les plantes. Des séquences répétitives d'ADN peuvent générer des bandes FISH sur des chromosomes individuels pour fins de caryotypage et d'analyse phylogénétique. Employée sur des chromosomes méiotiques au stade pachytène et couplée à l'emploi de systèmes d'imagerie numérique, l'analyse FISH est devenue une méthode efficace pour développer des cartes physiques chez les espèces végétales. Appliquée à des fibres étirées d'ADN, l'analyse FISH fournit une approche à haute résolution pour analyser de grandes molécules d'ADN et pour caractériser de grands locus génomiques. La cartographie physique par FISH se veut une approche complémentaire précieuse pour le séquençage des génomes et le clonage positionnel. Les auteurs prédisent que la FISH continuera de jouer un rôle important permettant de relier l'information sur des séquences d'ADN et la biologie des chromosomes. La FISH, couplée à des méthodes immunologiques, sera de plus en plus employée pour étudier les caractéristiques de la chromatine au niveau cytologique, lesquelles contribuent à l'expression et à la régulation des gènes.

Mots clés : FISH, biologie du chromosome, recherche génomique, cytogénétique moléculaire.

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Introduction

The development of the DNA in situ hybridization technique (Gall and Pardue 1969; John et al. 1969) marked the transition from the classical cytogenetics era to the modern molecular cytogenetics era. The basic procedure of in situ hybridization is the labeling of a DNA probe and hybridiza-

tion of said probe to cytological preparations. Radiation-based methods were used in probe labeling and signal detection in early techniques. However, such methods were soon replaced by fluorescence-based techniques (Langer-Safer et al. 1982). Fluorescence in situ hybridization (FISH) techniques have continued to evolve in the last 20 years and have played a key role in the development of modern molecular cytogenetics. Early developments of the FISH techniques and their application in plant genome mapping were reviewed in our previous paper (Jiang and Gill 1994). Here, we review developments in the same research field made during the intervening years.

Technical developments

Although the principle steps of the FISH procedure have stayed the same, various technical modifications have been adapted in plant cytogenetics labs. Several of these technical developments are discussed in this paper.

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Tyr-FISH

Detecting FISH probes as small as a typical gene of 1–3 kb in size has been one of the major goals for many plant cytogeneticists. Numerous methods have been developed to improve the detection sensitivity of FISH experiments. One of the signal amplification techniques is to use a peroxidase-conjugated antibody as the first layer of signal detection, then to use fluorochrome-labelled tyramides as peroxidase substrates to generate and deposit many fluorochromes close to the in situ bound peroxidase (Raap et al. 1995). The sensitivity of FISH can be increased by 10–100 times using this signal amplification system (van Gijlswijk et al. 1997). DNA probes smaller than 1 kb were successfully visualized on plant chromosomes using Tyr-FISH (Khrustaleva and Kik 2001; Stephens et al. 2004). However, any signal amplification system may also significantly enhance the background signal. Such techniques often need to be adjusted in individual labs to reach the optimum signal-to-noise ratio. The quality of the DNA probes and the cytological preparations are other factors that should be considered for optimum results.

Three-dimensional FISH using optical-sectioning microscopy

A three-dimensional FISH technique was developed in maize by Bass et al. (1997). Meiotic cells are lightly fixed in a buffer designed to preserve chromosome structure. Pollen mother cells are then gently extruded out of fixed anthers and embedded in optically clear polyacrylamide for staining and imaging. Stacks of FISH images are taken and composed into a single three-dimensional image. Individual chromosomes bearing the FISH signals can be traced and computationally straightened (Harper and Cande 2000; Koumbaris and Bass 2003). Since the chromosome structure is well preserved using this technique, presumably the advantage of this technique is the precise location of DNA probes on chromosomes within the nucleus. However, three-dimensional FISH imaging by deconvolution or confocal microscopy is considerably more expensive than a conventional fluorescent microscope. Wang et al. (2006) recently reported that pachytene chromosomes prepared from the conventional squash method showed a karyotype similar to that prepared from three-dimensional chromosome preparations and are more suitable for mapping single-copy DNA sequences. Thus, the three-dimensional FISH system does not offer major advantages for mapping DNA probes on chromosomes, but it is valuable for investigating the spatial organization of DNA sequences within the nucleus and for detecting proteins in immunoassays because of its mild fixation process and preservation of chromatin structure.

FISH on super-stretched chromosomes

Flow-sorted plant chromosomes at mitotic metaphase can be stretched to more than 100 times their original size after a mild proteinase-K digestion (Valarik et al. 2004). FISH on stretched chromosomes showed brighter signals than on the untreated control presumably as a result of better probe accessibility to the stretched chromatin (Valarik et al. 2004). FISH on super-stretched metaphase chromosomes provides a mapping resolution of up to 70 kb (Valarik et al. 2004), similar to the resolution on meiotic pachytene chromosomes

(Cheng et al. 2002a). Thus, super-stretched metaphase chromosomes provide an alternative FISH mapping target for those plant species where meiotic pachytene chromosomes are not suitable for cytological analysis. However, this method requires a chromosome-sorting facility and is only useful for species in which some or all of the chromosomes can be individually sorted.

FISH on DNA fibers

High-molecular-weight genomic DNA or individual DNA molecules from large-insert DNA clones can be spread on glass slides for FISH analysis (Fransz et al. 1996a; Jackson et al. 1999). DNA prepared from bacterial artificial chromosome (BAC) clones or plant tissues extends approximately 2.5–3.5 kb/ μm on slides (Fransz et al. 1996a; Jackson et al. 1999; Cheng et al. 2002a). Thus, the fiber-FISH method provides fine-mapping resolution of up to a few kilobases. Fiber-FISH has been used in various types of plant genome mapping projects, including analysis of structure and organization of repetitive DNA sequences (Fransz et al. 1996a; Dong et al. 1998; Jackson et al. 1998; Miller et al. 1998; Pich and Schubert 1998; Zhong et al. 1998; Ohmido et al. 2000; Fukui et al. 2001; Gindullis et al. 2001; Cheng et al. 2002b); mapping of a single, large, genomic locus (Stupar et al. 2001; Adawy et al. 2004; Tek et al. 2005); mapping of BAC (Jackson et al. 1999; Yuan et al. 2002; Nagaki et al. 2003; Lin et al. 2005) and chloroplast DNA molecules (Lilly et al. 2001); and analysis of transgenic DNA loci (Wolters et al. 1998; Jackson et al. 2001; Svitashv and Somers 2001; Nakano et al. 2005). Fiber-FISH is an effective method to measure the size of gaps in physical maps (Jackson et al. 1998). This approach was used to measure most of the remaining physical gaps in the rice sequence maps (Feng et al. 2002; Sasaki et al. 2002; Yu et al. 2003).

There are two major technical difficulties that may limit the application of the fiber-FISH method: (i) if a large-insert DNA clone, such as a BAC clone, contains an extensive amount of repetitive DNA sequences, signals from such a clone may be difficult to locate on genomic DNA fibers even with the application of blocking DNA, thus, most BAC clones from species with very large genomes, such as wheat, are not suitable for fiber-FISH analysis; and (ii) although fiber-FISH signals can be produced from DNA probes as small as <1 kb, it is difficult to distinguish short fiber-FISH signals from background signals, thus, small DNA probes can be identified only together with long adjacent reference signals. In addition, the lengths of the Fiber-FISH signals from a specific probe may vary significantly owing to the different degree of DNA extension. Thus, a large number of signals and statistical analysis are required to obtain accurate measurements.

FISH as a tool for chromosome identification

A robust method for chromosome identification is the most important foundation for the success of cytogenetics research. Unfortunately, such a method is not available for many plant species, especially those with small chromosomes. FISH signals derived from a single repetitive DNA probe or a cocktail containing several DNA probes can pro-

vide a hybridization pattern that allows identification of all chromosomes within a species. Since different probes or probe cocktails can be developed for a particular species, the FISH-based chromosome identification method is more versatile than the traditional chromosome banding techniques. More importantly, FISH-based chromosome identification systems can be integrated directly into FISH mapping of other DNA sequences.

Many repetitive DNA elements generate specific FISH signal patterns on individual chromosomes within a single species (Fuchs et al. 1994; Busch et al. 1995; Tsujimoto et al. 1997; Navratilova et al. 2003; Koo et al. 2005). If the FISH signals from a single repetitive DNA probe are not informative enough to distinguish every chromosome, a combination of two or more repetitive DNA probes can be labeled and used as a cocktail to increase the resolving power. For example, FISH signals derived from two repetitive DNA probes allowed identification of all 21 chromosomes in hexaploid wheat (Pedersen and Langridge 1997). Similar repetitive DNA probe cocktails have been developed in several plant species (Fransz et al. 1998; Sadler and Weber 2001; Hizume et al. 2002; Vischi et al. 2003; Kato et al. 2004; Koo et al. 2004; Lengerova et al. 2004) (Fig. 1A). One potential drawback of using repetitive DNA probes is the polymorphism of the FISH signal patterns among different varieties and accessions, especially for open-pollinated plant species. The polymorphism can potentially interfere with the identification of the same chromosomes in different lines.

As an alternative for repeat-based FISH probes, chromosome-specific cytogenetic DNA markers (CSCDM) can be developed for individual chromosomes using large-insert genomic DNA clones, such as BACs (Dong et al. 2000). A set of CSCDMs can be developed to generate a unique pattern of FISH signals that allow identification of all chromosomes. Kim et al. (2002) demonstrated such a strategy involving simultaneous identification of all 10 sorghum chromosomes using a set of 22 BAC clones. BACs can be isolated using DNA markers that have been mapped on genetic linkage groups. Thus, chromosomes identified by CSCDMs can be integrated with genetic linkage groups. This approach has been successfully used to integrate genetic linkage groups with chromosomes in several plant species (Dong et al. 2000; Cheng et al. 2001a; Kulikova et al. 2001; Howell et al. 2002; Kim et al. 2002; Pedrosa et al. 2002; Kim et al. 2005b; Zhang et al. 2005).

There are several advantages for using CSCDMs in chromosome identification. First, it gives each chromosome a distinctive feature to be used to differentiate it from the rest of the chromosomes. It is often difficult to distinguish a specific chromosome from the rest of the chromosomes within the same cell in other chromosome identification systems. Second, the quality of the chromosome preparations is not important for chromosome identification using this system, but it is often critical in other systems. Third, this system can be applied to most plant species, especially those with large numbers of small chromosomes. Genetic linkage maps and BAC libraries have been developed in most crop and model plant species. Thus, CSCDMs can be readily developed in these species. However, most BACs from species

with very large genomes, such as wheat, do not generate unique locus-specific FISH signals (Zhang et al. 2004a). It will be a challenge to develop CSCDMs in such plant species.

FISH-based karyotyping and phylogenetic analysis

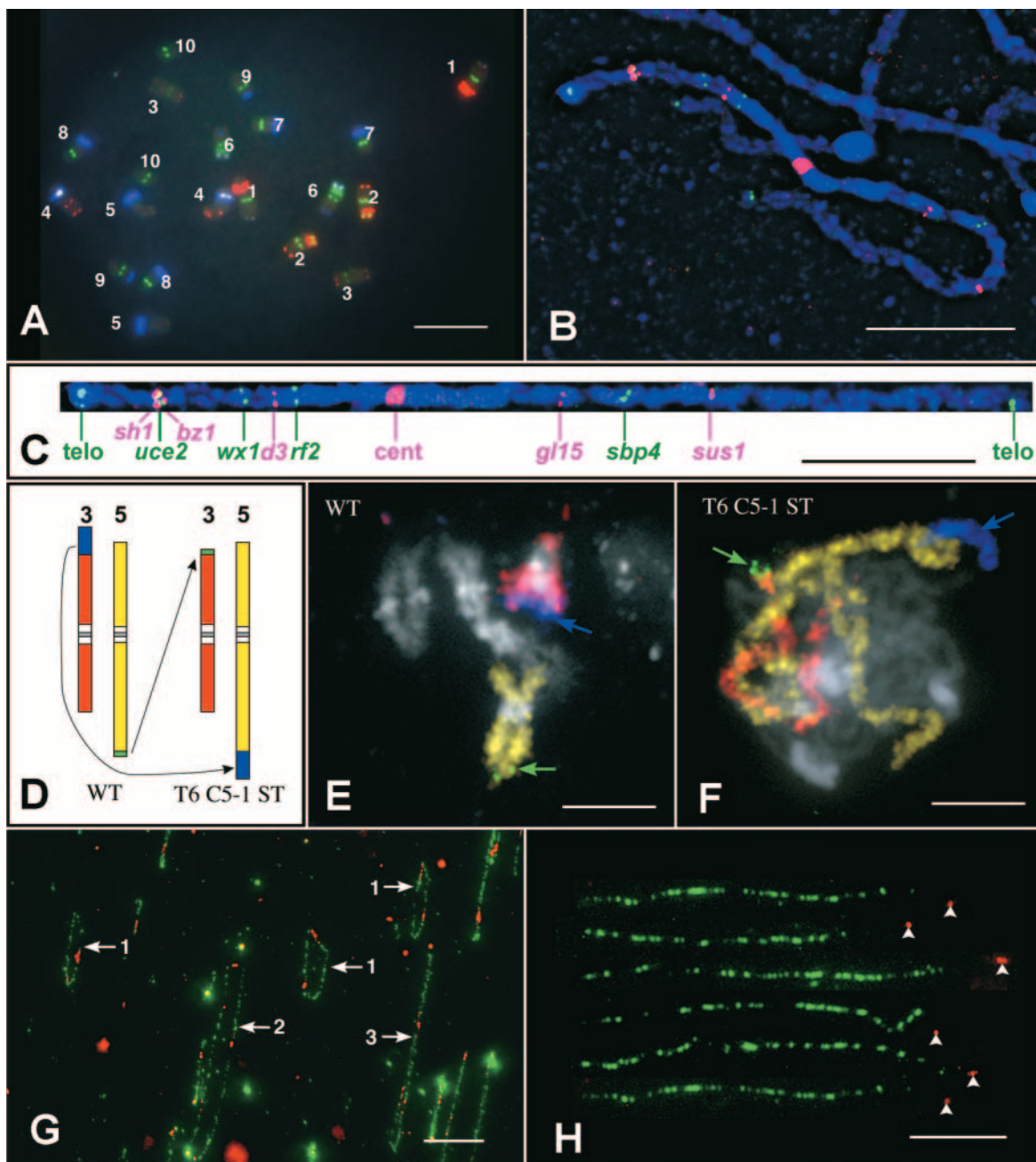
FISH-based chromosome identification systems can be used for karyotyping. For example, several repetitive DNA probes generate specific hybridization patterns on chromosomes of wheat and its related species (Mukai et al. 1993; Pedersen and Langridge 1997). The FISH signal patterns derived from these probes produce a unique and stable FISH karyotype for each species (Badaeva et al. 1996, 2002). The FISH karyotypes from some repetitive DNA probes are similar to karyotypes based on C- or N-banding analysis (Cuadrado et al. 1995; Pedersen and Langridge 1997). Thus, FISH-based karyotyping provides an evolutionary and phylogenetic view of related plant species (Lim et al. 2000; Hizume et al. 2002). Repetitive DNA sequences are likely to have evolved under different evolutionary pressures as compared with functional genes. Thus, phylogenetic schemes derived from repeat-based comparative FISH karyotyping offer an independent test of molecularly based phylogenies (Lim et al. 2000).

Badaeva et al. (1996, 2002) developed FISH karyotypes of several diploid and polyploid *Triticum* and *Aegilops* species using a number of repetitive DNA probes. Comparison of the FISH karyotypes of these species provided chromosomal evidence of the evolutionary relationship between these species (Badaeva et al. 1996, 2002). Karyotyping using repetitive DNA probes can also visualize intergenomic chromosome translocations in polyploid species (Linc et al. 1999; Zhang et al. 2004b). Tobacco (*Nicotiana tabacum*, S5TT) is a well-established allotetraploid. The maternal genome (SS) donor of tobacco is derived from *N. sylvestris*. The paternal genome (TT) donor was less clear; however, comparative FISH karyotyping of several *Nicotiana* species using a number of repetitive DNA probes demonstrated *N. tomentosiformis* to be the T-genome donor (Lim et al. 2000; Murad et al. 2002).

Chromosome-specific painting and its application in genome research

Unlike GISH (genomic in situ hybridization), which is a widely used technique for genome-specific chromosome painting in hybrids and polyploid species (reviewed in Jiang and Gill 1994), chromosome-specific painting has been a difficult technique to apply in plants. In mammalian species, DNA probes covering an entire chromosome can be developed using chromosome sorting or microdissection-based methods. Such chromosome-painting probes then can be used to visualize a single chromosome among related species. Comparative chromosome painting has been a powerful tool to study the synteny and chromosomal evolution in primates and other mammals (Wienberg and Stanyon 1997). However, in most plant species, chromosome-sorting-derived "chromosome-specific probes" contain extensive amounts of repetitive DNA sequences that are difficult to block in FISH procedures (Fuchs et al. 1996). In our laboratory, we

Fig. 1. Applications of FISH in plant genome research. (A) Identification of all 20 somatic metaphase chromosomes of maize (line W22) by FISH using a cocktail of 9 repetitive DNA probes (Kato et al. 2004). Photo by A. Kato and J.A. Birchler. (B) Simultaneous FISH mapping of 9 single-copy sequences, as well as the centromere and telomere markers on pachytene chromosome 9 of maize (Wang et al. 2006). (C) A computationally straightened chromosome 9 from the same image shown in B. Photos by R. Wang and Z. Cande. (D, E, and F) Four-color chromosome painting using differently labeled BAC contigs that cover *Arabidopsis thaliana* chromosomes 3 and 5. The painting results confirmed a reciprocal translocation reported in a transgenic line T6 C5-1 ST (Aufsatz et al. 2002). (D) Schematic presentation of chromosomes 3 and 5 in wild-type (WT) and transgenic translocation line T6 C5-1 ST. (E) Painting of diplotene chromosomes 3 and 5 of wild-type. An arrow points to the translocated segments illustrated in D. (F) Painting of pachytene chromosomes 3 and 5 of the transgenic translocation line T6 C5-1 ST. An arrow points to the translocated segments illustrated in D. Photos by A. Pecinka and I. Schubert. (G) FISH mapping of chloroplast DNA molecules (Lilly et al. 2001). A single microscopic field showing several chloroplast molecules from tobacco. Arrows marked with the numeral "1" point to 3 open-circle monomeric molecules; an arrow marked by a numeral "2" points to an open-circle dimeric molecule; an arrow marked by a number "3" points to a partially open-circle dimeric molecule. Photo by J. Lilly. (H) Fiber-FISH mapping of telomere (red) and BAC b0026K20 (116 kb, green), which is located at the distal end of the long arm of rice chromosome 11 (Rice Chromosomes 11 and 12 Sequencing Consortia 2005). The telomeric signals from 6 independent signals are indicated by arrowheads. The estimated gap that separated the BAC and the telomere was 22 ± 3.8 kb. Photo by W.W. Jin. Bars represent 5 μ m in E and F; 10 μ m in A, B, C, G, and H.



failed to generate chromosome-specific signals from probes derived from flow-sorted tomato chromosomes (J. Jiang, unpublished data).

Lysak et al. (2001) demonstrated that the relatively small chromosomes of *Arabidopsis thaliana* can be painted by labeling pools of more than 100 BAC clones spanning an entire chromosome arm (Figs. 1D–1F). This technique was used to track individual *Arabidopsis* chromosomes in the interphase nuclei and to reveal the spatial arrangement and functional properties of individual chromatin domains (Fransz et al. 2002; Pecinka et al. 2004). Using this approach, Fransz et al. (2002) demonstrated that the euchromatin portion of the *Arabidopsis* genome is organized as loops spanning 0.2–2 Mb, which emanate from the condensed centromeric chromocenters (CC). CC and loops together form a chromosome territory with homologous CCs and territories associated frequently. Probes specific to an *Arabidopsis* chromosome and a chromosomal segment can be used to paint chromosomes from species related to *A. thaliana* (Lysak et al. 2005, 2006). Such comparative painting analysis provides an efficient and powerful approach to visualize the genome duplications and karyotype evolution of the species that have had limited genetic and cytogenetic studies (Lysak et al. 2005, 2006).

The *Arabidopsis* genome is largely euchromatic and the heterochromatin is concentrated in the centromeric regions. Thus, the success of chromosome-specific painting in *Arabidopsis* is due to the elimination of highly repetitive DNA sequences in the painting probes by excluding BACs containing such sequences. Whether or not the same approach can be applied to other plant species remains to be seen. Success of this technique in other plants may rely on development of methods that allow preferential elimination of highly repetitive DNA sequences in the painting probes.

FISH and physical mapping

One of the most important applications of FISH techniques has been their use as physical mapping tools. The traditional photographic system was time consuming and expensive when processing large amounts of FISH data. Thus, early FISH techniques coupled with the photographic system was used to map only a limited number of clones on chromosomes. However, the digital imaging system using CCD (charge coupled device) cameras now enables cytogeneticists to collect and process large amounts of FISH data, thus allowing application of the FISH technique to large-scale physical mapping projects.

Detection of small probes by FISH

How small a probe can you visualize with FISH is one of the most common questions posed to plant cytogeneticists. Various technical modifications have been proposed by different laboratories to improve FISH sensitivity. Many laboratories have developed in-house tricks to enhance detection sensitivity. The lower limit in most of the reports appears to be around 1–3 kb (Fransz et al. 1996b; Ohmido et al. 1998; Desel et al. 2001; Khrustaleva and Kik 2001; Stephens et al. 2004; Wang et al. 2006) (Figs. 1B–1C). Probes <1 kb were detected in some reports (Desel et al. 2001; Khrustaleva and Kik 2001; Stephens et al. 2004). However, small probes are

generally detected at low frequencies and results may be inconsistent for different probes. In addition, such results are often not reproducible in different laboratories using the same technique.

One approach to circumvent the difficulty for detecting small DNA probes is to use large-insert genomic DNA probes that are anchored by the targeted small DNA probes. Various types of genomic DNA clones have been used in FISH mapping, including λ clones (Peterson et al. 1999), cosmid clones (Sadder and Weber 2002), yeast artificial chromosome (YAC) clones (Zhong et al. 1999; Fransz et al. 2000), and BAC clones (Jiang et al. 1995; Lapitan et al. 1997; Zwick et al. 1998; Zhong et al. 1999; Tor et al. 2002; Schnabel et al. 2003). One potential problem is that the large-insert genomic clones, especially those from species with very large genomes, may contain an extensive amount of repetitive DNA sequences that could prevent the localization of the single-copy sequences. The cross hybridization from the repetitive DNA sequences can be minimized by pre-annealing the probe with *C₀t1* or sheared genomic DNA. However, for genomic clones that contain a high percentage of repetitive sequences, the cross-hybridization may not be fully overcome by this approach. Zhang et al. (2004a) were unable to produce locus-specific signals in a sample of 56 BACs identified using unique-copy RFLP probes. Koumbaris and Bass (2003) demonstrated that sorghum BACs selected by hybridization with genetically mapped maize probes are a valuable resource in the development of physical maps of maize because sorghum and maize do not share the most abundant repetitive DNA families.

The resolving power of FISH as a physical mapping tool

Another common question posed to plant cytogeneticists is what is the resolution of FISH mapping. The resolving power of FISH depends on the cytological targets, currently including interphase nuclei, mitotic prometaphase and metaphase chromosomes, super-stretched mitotic metaphase chromosomes, meiotic pachytene chromosomes, and extended DNA fibers. Mitotic metaphase chromosomes are the most common cytological target for FISH mapping because root tips are readily available for most plant species. However, such highly condensed chromosomes have the lowest resolving power for FISH mapping. Pedersen and Linde-Laursen (1995) suggested that a minimum of 5–10 Mb distance would be necessary to resolve FISH signals of two DNA clones on barley metaphase chromosomes (Pedersen and Linde-Laursen 1995). On less-condensed prometaphase chromosomes, the FISH resolving power can be increased to 2 Mb (Cheng et al. 2002a). However, chromatin condensation varies widely for different parts of prometaphase chromosomes. Thus, the resolving power of prometaphase FISH will depend on the location of the targeted clones and stage (early or late prometaphase) of the target chromosome.

Interphase nuclei, super-stretched mitotic metaphase chromosomes, and meiotic pachytene chromosomes provide intermediate resolving power for FISH mapping. The relative positions of clones separated by <100 kb can be resolved on these cytological targets (Jiang et al. 1996; de Jong et al. 1999; Cheng et al. 2002a; Valarik et al. 2004; Wang et al. 2006). Pachytene chromosomes are particularly versatile tar-

gets for FISH mapping. Late pachytene chromosomes can be used to orient the telomere–centromere positions of the adjacent clones, whereas early pachytene chromosomes can be used to resolve even partially overlapped BAC clones (Cheng et al. 2002a). Nevertheless, pachytene chromosomes are not amenable for cytological analysis in many plant species. In these species, cytological targets other than pachytene chromosomes need to be considered to resolve closely linked clones. Fiber-FISH provides the highest resolution. DNA clones separated by few kilobases can be resolved on DNA fibers (Figs. 1G–1H).

Development of FISH-based physical maps

Currently there are 3 general approaches to develop a physical map that covers an entire chromosome or an entire genome: (i) DNA contigs can be assembled by fingerprinting large-insert genomic DNA clones, such as BACs; (ii) genetically mapped DNA markers can be physically mapped to specific chromosomal segments using cytogenetic stocks, such as deletion stocks or translocation stocks; and (iii) DNA clones can be mapped directly on chromosomes using FISH. Each of these three approaches has its own strengths and weaknesses. The strengths of the FISH-based approach include the ability to visualize the chromosomal location of each DNA clone, the direct assignment of mapped clones to chromosomal regions associated with heterochromatin or euchromatin, and the potential to construct a map within a short period of time at a relatively low cost.

FISH mapping of BACs anchored with genetically mapped DNA markers is a very efficient way to construct a FISH-based physical map. Such an approach would fully integrate the genetic linkage maps with chromosomal maps. Cheng et al. (2001a) demonstrated this approach by FISH mapping of 18 marker-anchored BAC clones on the pachytene chromosome 10 of rice. This FISH mapping effort revealed the distribution of genetic recombination along the entire length of rice chromosome 10 and the genetic and physical locations of the centromere (Cheng et al. 2001b). Similar FISH-based physical maps have been developed in several other plants, including sorghum (Islam-Faridi et al. 2002; Kim et al. 2005a, 2005c), maize (Koumbaris and Bass 2003), *Brassica oleracea* (Howell et al. 2005), soybean (Walling et al. 2006), and rice chromosome 5 (Kao et al. 2006). A high-quality pachytene chromosome preparation may be reprobbed several times with different probes (Cheng et al. 2001b); alternatively, multiple probes can be mapped onto the same preparation (Islam-Faridi et al. 2002; Wang et al. 2006) (Figs. 1B–1C). These methods can significantly increase the efficiency and accuracy of measurements of the FISH signals.

FISH is also a valuable complementary mapping approach to BAC contig development. BAC contigs can be anchored to a euchromatic or a heterochromatic region by FISH (Fransz et al. 2000; Cevik and King 2002; Budiman et al. 2004). The orientations of adjacent BAC contigs can be determined by anchoring contigs to chromosomes using FISH. Such information would be invaluable if the BAC contigs were developed for map-based gene-cloning projects. Physical distances between adjacent BAC contigs can be measured by fiber-FISH analysis (Jackson et al. 1998; Feng et al. 2002; Sasaki et al. 2002; Yu et al. 2003; van der Knaap et al.

2004). The information on gap sizes is important for developing appropriate strategies to eventually close the gaps.

Comparative FISH mapping

DNA clones from one species can be used as probes for FISH mapping in a related species. Such a comparative FISH mapping approach has several advantages over traditional comparative genetic linkage mapping, i.e., (i) a mapping population is not required; (ii) FISH mapping does not rely on polymorphism, thus any clones from one species can be used as a FISH probe as long as they generate signals in another species; and (iii) some evolutionary rearrangements between the two species, such as duplications, can be readily visualized.

Several labs have reported FISH mapping of *A. thaliana* BACs on chromosomes of *Brassica* species (Jackson et al. 2000; Ziolkowski and Sadowski 2002; Howell et al. 2005). *Arabidopsis* and *Brassica* species diverged from a common ancestor approximately 15–20 million years ago (Yang et al. 1999). Most *Arabidopsis* BAC clones generate weak but detectable FISH signals in *Brassica* species. Comparative FISH mapping between *Arabidopsis* and *Brassica* species provided a direct visualization of the genetic colinearity between the two species and genome duplications within *Brassica* species (Jackson et al. 2000; Ziolkowski and Sadowski 2002; Howell et al. 2005; Lysak et al. 2005). The main technical difficulty of the comparative FISH mapping approach is that a DNA clone, such as a BAC, generates strong FISH signals in one species, from which the clone was originated, but produces weak or no signals at all in a related species. The hybridization stringency for FISH, which has only one target on a chromosome, is much more difficult to manipulate than the stringency for gel-blot hybridization with thousands of targets on the blot. Generally, the longer the phylogenetic distance between the two species, the weaker the cross-hybridization FISH signals (Zwick et al. 1998; Hasterok et al. 2006). For example, many sorghum BACs produce usable FISH signals on maize chromosomes (Zwick et al. 1998; Koumbaris and Bass 2003), but produce much weaker or no signals on rice chromosomes (Zwick et al. 1998).

GISH-based physical mapping

King et al. (2002) demonstrated a GISH-based approach for physical mapping. In this approach, the recombination between a chromosome from *Festuca pratensis* and a chromosome from *Lolium perenne* was visualized using the GISH method (King et al. 2002). The single *F. pratensis* chromosome was divided into 18 segments that are included in different recombinant chromosomes. Genetically mapped DNA markers were then mapped to specific chromosome segments. A similar approach has also been used for integration of genetic and physical maps of two *Allium* chromosomes (Khrustaleva et al. 2005). This GISH-based mapping strategy is similar to physical mapping using deletion and translocation stocks. This approach overcomes the major drawback of the tedious and time-consuming process of developing a large number of deletion and translocation stocks. However, the GISH-based method can be applied to analyze recombination only between partially homologous chromosomes using interspecific or intergeneric crosses.

Future outlook

FISH as a physical mapping tool

DNA-sequencing technologies continue to develop at a fast pace. In the foreseeable future, whole-genome sequencing will no longer be a hurdle for any plant species. However, this does not spell the end of FISH as a physical mapping tool. FISH will continue to play a role in relating sequence information to chromosome biology as a part of the integrated genome-mapping approach. FISH is the only method to anchor DNA sequences to specific euchromatin and heterochromatin features on chromosomes. All of the currently available DNA sequencing technologies will inevitably result in physical gaps. Information on the size of the gaps is critical for designing appropriate gap-closing strategies. FISH has proven to be the most efficient methodology to map the locations and sizes of such gaps (Fig. 1H). The resolving power of genetic linkage mapping will be limited for genomic regions in which the genetic recombination is severely suppressed. FISH is a valuable approach to delineate and characterize such non-recombinant regions (Akiyama et al. 2004; Goel et al. 2006).

Genomic regions consisting of long tracks of highly repetitive DNA sequences are extremely difficult or impossible to be sequenced and to be analyzed by gel-blot-based approaches. For example, the centromeres in most multicellular eukaryotes contains megabase-sized satellite arrays (Jiang et al. 2003). Thus, the centromeres have been left as gaps in the sequence maps in most model eukaryotes. Similar situation exists for chromosome ends, as telomeric ends are often difficult to clone. FISH has been and will continue to be a powerful technique to delineate the structure and DNA composition of such genomic regions (Gindullis et al. 2001; Stupar et al. 2001; Jin et al. 2004, 2005; Tek and Jiang 2004; Lamb et al. 2005).

Immuno-FISH

A biological question may not be answered by a simple localization of DNA sequences in nuclei or on chromosomes. However, physical localization of a DNA sequence together with its associated protein may dramatically enhance the power of FISH. Several plant laboratories have developed techniques that combine FISH with immunoassay methods (Jasencakova et al. 2000, 2001; Wako et al. 2002; Zhong et al. 2002; Probst et al. 2004; Lamb et al. 2005). In most published protocols, immunoassays using specific antibodies were performed first, and cytological preparations were then postfixed and followed with the FISH procedure. If the immunoassay signals are significantly deteriorated by the post FISH procedure, they can be recorded independently before FISH. Immuno-FISH has been used to reveal the association of histone modifications with specific genomic regions (Jasencakova et al. 2000, 2001; Wako et al. 2002; Probst et al. 2004) and association of centromere-specific proteins with specific repetitive DNA sequences (Zhong et al. 2002; Lamb et al. 2005; Nasuda et al. 2005). Immuno-FISH can also be performed on stretched chromatin, which significantly increases the resolving power to reveal interactions between DNA and proteins (Jin et al. 2004, 2005; Shibata and Murata 2004).

Application of FISH in fundamental research

FISH will be increasingly used as a tool to answer fundamental biological questions (Tessadori et al. 2004). Since FISH can be used to associate a DNA sequence with a specific chromosomal feature or a chromatin feature in the interphase nuclei in either two or three dimensions, it is particularly useful to study the association of gene expression/regulation with epigenetic features at the chromatin level (Tessadori et al. 2004; Wegel et al. 2005). Recently, Costa and Shaw (2006) conducted elegant three-dimensional FISH experiments on intact root epidermal tissue to reveal the association of chromatin structure with gene expression and cell differentiation. The experimental condition of FISH in this study was controlled in such a way that a strong hybridization signal was correlated with an "open" chromatin structure that is easily accessible to the FISH probe, whereas faint or no hybridization was correlated with a "closed" chromatin structure that is not accessible to the FISH probe (Costa and Shaw 2006). FISH results clearly demonstrated that the open or closed chromatin structure around the homeodomain transcription factor *GL2* is required for position-dependent cell specification (Costa and Shaw 2006).

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