

Fluorescent In Situ Hybridization (FISH) on Pachytene Chromosomes as a Tool for Genome Characterization

René Geurts and Hans de Jong

Abstract

A growing number of international genome consortia have initiated large-scale sequencing projects for most of the major crop species. This huge amount of information not only boosted genetic and physical mapping research, but it also enabled novel applications on the level of chromosome biology including molecular cytogenetics that supports plant genetics, genomics, and breeding programs. The simultaneous detection of a large number of BAC-based probes by multicolor fluorescent in situ hybridization (FISH) can provide a rapid overview of super-contig and gap distribution on euchromatin chromosome areas and will display directly and precisely the positions of chromosome rearrangements. Furthermore, hybridizations of BACs on the chromosomes of related species can confirm genomic colinearity, or the occurrence of inversions and translocations events. This cross-species FISH together with meiotic pairing studies is a powerful source of information that elucidates the nature of genome rearrangements, and the consequences of such rearrangements for introgressive hybridizations. In this chapter we describe a general-purpose protocol for FISH on pachytene chromosomes.

Key words Fluorescent in situ hybridization, FISH, Pachytene, BAC-FISH painting

1 Introduction

Whole-genome sequencing has revolutionized biological sciences. While large consortia laboriously sequenced most model organisms, more recent sequencing efforts made genome information available for almost every important eukaryotic organism. In the field of legumes (*Fabaceae*) the genomes of soybean (*Glycine max*), *Medicago truncatula*, *Lotus japonicus*, and pigeon pea (*Cajanus cajan*) are already made available and there are possibly many more to come [1–4]. However, while sequencing itself has become very effective and relatively easy, sequence assembly has not become easier. One of the tools that are helpful in the assembly is the chromosomal mapping of single copy and repetitive sequences by fluorescent in situ hybridization (FISH) [5–8].

FISH provides the possibility to determine the physical position of a certain sequence on a chromosome. In FISH, DNA from a clone is fluorescently labeled and used as probe to identify the location of the specific sequence on a set of metaphase chromosomes or pachytene bivalents, the latter giving on average a 20-fold higher spatial resolution [9]. Thus an approximate genomic position can be determined for each individual clone. The probe DNA can vary widely in size. Most often labeled fragments of 50–150 kb genomic DNA (e.g., a bacterial artificial chromosome (BAC) clone) are used as probes for hybridization on chromosomal targets. BAC libraries are generally available for model species and for most of the important crops. Besides such insert clones, small DNA fragments of even 500 bp can be used as a probe instead. In such cases the detection will be indirect through application of a signal amplification step using specific detection amplification systems. A disadvantage of using large probes, like BAC clones, can be the presence of tandem or dispersed repeats in the sequence, which will hybridize to multiple loci, and so can cause erratic mapping of the unique sequences. To block repeats from hybridization, an excess of repeat sequences is added to the hybridization mix. The most accepted method in plant science is Cot DNA, a pool of highly and middle repetitive DNA obtained from sheared single-strand genomic DNA and reannealed according to the principles of DNA reassociation kinetics [10]. Cot DNA is used to study genome structure and organization and has also been used to simplify the sequencing of genomes that contain large amounts of repetitive sequence [11]. In this way repeats can be effectively suppressed in the labeled probe, allowing the localization of almost any genomic clone in a medium-sized plant genome.

Here, we describe a general protocol for FISH on pachytene chromosome bivalents using young flower buds as starting material. The advantage of this procedure is that besides chromosome complements at meiotic pachytene that can be observed, also chromosome sets at mitotic (pro-)metaphase are abundantly present, which can be used simultaneously for analyses. Additionally, a protocol is included for masking repeat sequences with Cot-DNA pre-hybridization.

2 Materials

2.1 *Flower Buds for Chromosome Preparations*

1. Anthers from young flower buds.
2. Fixative: Glacial acetic acid:ethanol 98 % (1:3).
3. 70 % ethanol.
4. Grease-free microscope slides, 24×24 mm coverslips, clean slide boxes.
5. 45 and 60 % acetic acid.

6. Counterstaining solution: 1 % carmine in 45 % acetic acid.
7. For the sodium citrate buffer we add 10 mM citric acid to 10 mM sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, which acts as the base) until a pH of 4.5 was obtained.
8. Proteolytic enzymes: Cytolhelicase, pectolyase Y-23, and cellulase RS (Sigma). An enzyme mixture is made (1 % w/v each) in the sodium citrate buffer.
9. Microscope with high-resolution (N.A. > 0.5) phase contrast optics.

2.2 DNA Isolation and Labeling

1. Plasmids containing DNA repeat sequences: For example, 5S rDNA (e.g., clone pCT4.2 [12]), 45S rDNA (e.g., clone pTA71 [13]), and/or telomere repeats (e.g., clone pAtT4 [14]).
2. Plasmids containing DNA that will be used as probe (e.g., BAC clones).
3. Plasmid DNA is isolated with a plasmid DNA isolation kit (mini and midiprep) (e.g., Qiagen).
4. Biotin-dUTP and digoxigenin (Dig)-dUTP nick translation kits for labeling of the DNA clones (Roche).
5. For direct labeling of DNA Cy3.5-dCTP (GEHealthcare, gelifesciences.com), Fluorescein-12-dUTP (PerkinElmer), or CyTM3-dUTP (Amersham Biosciences, gelifesciences.com).

2.3 Cot-100 Isolation and Labeling

1. Genomic DNA isolated, e.g., by the CTAB method [15].
2. Sonicator (e.g., Virbra cell sonicator, www.ecomcat.co.uk).
3. Formamide.
4. A rotating oven.
5. DNA S1 nuclease.
6. Chloroform-isoamyl alcohol (24:1).
7. 100 % ethanol.
8. HB50 (50 % deionized formamide, 2× SSC, 50 mM sodium phosphate, pH 7).

2.4 FISH

1. Hybridization mixture: 20 ng labeled probe (plus 1 µg Cot-100 DNA if required), 10 µL 20 % dextran sulfate in HB50 (50 % deionized formamide, 2× SSC, 50 mM sodium phosphate pH 7, 20 % dextran sulfate).
2. Hot plate.
3. 50 % formamide/2× SSC three times, 5 min each.
4. Avidin-Texas Red to detect biotin-labeled DNA (Roche).
5. Biotin-conjugated goat-anti-Avidin to amplify biotin signal (Roche).

6. Sheep-antidigoxigenin-fluorescein (FITC) to detect Dig-labeled DNA probes (Roche).
7. Rabbit-anti-sheep-FITC to amplify Dig signal (Roche).
8. 5 µg/mL DAPI (4', 6-diamidino-2-phenylindole) in Vectashield antifade solution (Vector Laboratories).

2.5 Microscopy and Image Capturing

1. Microscope equipped with high N.A. plan apochromatics and epifluorescence illumination with filter sets for DAPI, FITC, and Cy3 fluorescence.
2. High-resolution camera, e.g., Photometrics Sensys 1,305 × 1,024 pixel CCD camera, which is controlled by Genus Image Analysis Workstation software (Applied Imaging Corporation).
3. Adobe Photoshop.
4. ImageJ processing software (<http://rsb.info.nih.gov/ij>).

3 Methods

3.1 Flower Buds for Chromosome Preparations

Chromosome slides can be made with cell spreads from different tissue types containing cell complements at different mitotic or meiotic stages. In most cases anthers are used containing pollen mother cells at pachytene, the stage in meiotic prophase I in which homologous chromosomes are fully paired. Pachytene chromosomes are always much longer than mitotic metaphase counterparts resulting in a significant higher resolution when compared to metaphase chromosomes. Also, pachytene chromosomes display well-differentiated patterns of heterochromatin and euchromatin, which is most helpful for identifying specific chromosome regions [9].

1. Plants should be grown under such conditions that they abundantly flower. To find anthers in the appropriate stage of development, young flower buds are collected in the late morning and directly fixed in freshly prepared glacial acetic acid:ethanol 98 % (1:3) and left in the fixative for a few hours. It is recommended to use at least 20× the amount of fixative than plant material. Refresh the fixative if the solution turns yellowish green. The plant material can be stored in the fixative at −20 °C for several days. If storage for a longer time is needed, the fixative should be replaced by ethanol 70 %. Now the samples can be stored at 4 °C. In this condition buds can be kept for months.
2. To stage the pollen mother cells containing meiocytes, young anthers are selected that are still transparent or light green. These anthers are squashed in a drop of 45 % acetic acid on a microscopic slide covered with a 24 × 24 mm coverslip.

Eventually a solution of 1 % carmine in 45 % acetic acid can be used to counterstain.

3. The developmental stage of the anthers is checked using a phase contrast microscope. Pollen mother cells at meiosis are recognized by a large cytoplasm and are surrounded by a clear callose wall. Anthers that are too early in development have no differentiated cells, whereas anthers that are too late in development contain pollen grains. As an example, for *Medicago truncatula* we found that anthers of 2 mm in length contain meiotic cells [16].
4. To prepare microscope slides containing chromosome spreads suited for hybridization flower buds in the appropriate stage of development are rinsed twice in Milli-Q water and 10 mM sodium citrate buffer (pH 4.5), and subsequently digested in an enzyme mixture of 1 % cytohelicase, 1 % pectolyase Y-23, and 1 % cellulase RS in the citrate buffer at 37 °C for 1–3 h [16]. Enzyme activity may decrease in time, so it is worth monitoring the progress of the enzymatic digestion of the material. Anthers that are sufficiently digested do not show remnants of cell walls and thick cytoplasm of the cells in the chromosome slides, whereas too long exposure to the pectolytic enzyme mixture will result in broken cells and affected chromatin structure.
5. After enzyme treatment, the soft flower buds are carefully rinsed with Milli-Q water and kept on ice for further treatment. Flower buds are transferred individually to clean grease-free microscopic slides. Supporting tissues are removed with dissecting needles as much as possible. Immediately thereafter 2 µL Milli-Q of water is added and the anthers carefully dissected and homogenized with fine needles.
6. The cells are macerated by adding 80 µL of 60 % acetic acid, mixed well, and spread over a small circle of 1–2 cm across the slide while carefully heating the slide on a hot plate for 2 min at 50 °C. Freshly prepared ice-cold acetic acid:ethanol (1:3) is added in a circle around the cells and the slides are left to dry.
7. Every slide (without coverslip) is carefully screened under the microscope with high-resolution (N.A. > 0.5) phase contrast optics to determine the developmental stage of the anther pollen mother cells, the quality of chromosome spreading, the absence of cytoplasm, and the presence of well-differentiated heterochromatin. Carefully selecting high-quality slides is most crucial for a successful FISH experiment. If needed, duration and temperature of the maceration and cell spreading should be adapted until satisfactory results are obtained. Only the best slides should be selected and can be stored in a dust-free microscopic box at 4 °C.

3.2 DNA Isolation and Labeling

In the start-up phase of FISH experiments a number of test experiments can be carried out with the ubiquitous 5S, 45S rDNA, and telomere repeats as probes. We generally use clone pTA71, which contains a 9.1 kb fragment of 45S rDNA of wheat [13], clone pCT4.2, which contains a 5S rDNA of *Arabidopsis thaliana* [12], and clone pAtT4, which contains a telomeric repeat 5'-CCCTAAA-3' of *A. thaliana* [14].

1. Plasmid DNA is isolated with a standard miniprep kit and labeled with either biotin-dUTP or Dig-dUTP by nick translation according to the manufacturer's manual.
2. For DNA isolation of BACs the alkaline lysate method is used [17] or any commercial plasmid DNA purification kit, e.g., for Midi preparation.
3. For indirect labeling, the isolated BAC DNA is labeled with either biotin-nick translation mix or Dig-nick translation mix. The biotin-labeled BAC probes are detected by Avidin-Texas Red and amplified by biotin-conjugated goat-anti-Avidin and Avidin-Texas Red. The Dig-labeled BAC probes are detected by sheep-anti-Dig-fluorescein (FITC) and amplified by rabbit-anti-sheep-FITC. For direct labeling, the isolated plasmid DNA is labeled with Cy3.5-dCTP, fluorescein-12-dUTP, or CyTM3-dUTP, without further detection and amplification steps.

3.3 Cot-100 Isolation and Labeling

The Cot-fraction of genomic DNA consists of highly repetitive tandem arrays (such as 45S rDNA, satellite repeats, and telomeres) and dispersed repeats (mostly LTR retrotransposons). In most plant species with small-to-moderate genome size such repetitive sequences will be enriched in the DNA Cot-100 fraction. Here we use a modified protocol described by Zwick et al. for preparing Cot-100 DNA [18]. The calculations should first be done for every new species. The Cot-DNAs thus obtained are first tested as a probe in FISH before using it as blocking agent.

1. Total genomic DNA is isolated using the cetyl trimethyl ammonium bromide (CTAB) method [15] and sonicated to fragment size of about 500 bp.
2. To produce Cot-100, the re-association temperature (T_{re}) has to be determined, which is: $T_{re} = T_m - 25$ (T_m : melting temperature). The T_m can be calculated by the following formula: $T_m = 81.5 + 16.6 \log M + 41 \times (\%G + C) - 500/L - 0.62F$ in which M =molar concentration of monovalent cations (=salt concentration), $\%G + C$ =G+C fraction of the genomic DNA, L =length of sheared DNA, and F =molar formamide concentration. The re-annealing time (T_s) should be calculated with the following equation: $T_s = 100/\text{molar (M) DNA concentration}$. The molar concentration of the DNA can be calculated using the average molecular weight for a deoxynucleotide monophosphate: 339 g/mol (see **Note 1**).

3. The sheared genomic DNA is denatured at 95 °C for 10 min, and re-annealed at 65 °C for the calculated time period in a rotating oven. The remaining single-strand DNA is removed using 1 U/μg DNA S1 nuclease at 37 °C for 90 min.
4. The reaction is stopped and extracted by adding equal volumes of chloroform-isoamyl alcohol (24:1), mixed well, and centrifuged at 2,250×*g* for 10 min. The upper layer is transferred into a new tube. DNA was precipitated with 2.5 volumes of ice-cold 100 % ethanol at -20 °C, overnight, and then centrifuged at 4 °C with 18,000×*g* for 30 min.
5. DNA is air-dried and dissolved in 20 μL HB50 (50 % deionized formamide, 2× SSC, 50 mM sodium phosphate, pH 7).

3.4 FISH

The FISH protocol as described here is largely based on the protocols previously described by Zhong et al. and Kuliková et al. with few adaptations [16, 19].

1. For FISH with the 5S rDNA, 45S rDNA, telomere, and other repetitive sequences, the DNA is labeled with dUTPs conjugated with a fluorophore (FITC, Cy3, etc.), which requires no further probe detection and signal amplification procedures.
2. In case of BACs used as probe, addition of Cot-100 DNA as a competitor for blocking repeats that are present in the BACs may be required. Cot-100 DNA is added in a 50:1 ratio compared to labeled probe.
3. A hybridization mixture contains 20 ng labeled probe (plus 1 μg Cot-100 DNA if required) and 10 μL 20 % dextran sulfate in HB50 (50 % deionized formamide, 2× SSC, 50 mM 1 M sodium phosphate pH 7, 20 % dextran sulfate), and is diluted with HB50 to a total volume of 20–40 μL.
4. The hybridization mix containing the probe is transferred onto the chromosome slide, denatured on a hot plate at 80 °C for 3 min, and incubated in a moist chamber at 37 °C for at least 20 h.
5. After hybridization the slides are rinsed in 50 % formamide/2× SSC three times, 5 min each.
6. Probes labeled with Dig-dUTP are detected with sheep-anti-Dig-fluorescein (FITC) and amplified with rabbit-anti-sheep-FITC, resulting in green signal. Probes labeled with biotin-dUTP are detected with streptavidin CY3 and amplified with streptavidin biotin, resulting in red signal. For the directly labeled probes, the detection and amplification steps are omitted.
7. The slides are washed three times in 70 %, 90 %, and 100 % ethanol, respectively, and air-dried. Chromosomes are counterstained with 5 μg/mL 4', 6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (*see* **Note 2**).

3.5 Microscopy and Image Capturing

1. Slides are studied using a microscope equipped with high N.A. plan apochromatics and epifluorescence illumination with filter sets for DAPI, FITC, and Cy3 fluorescence.
2. Images are captured by a CCD specially designed for fluorescent images.
3. Images are slightly improved by changing the thresholds for removing background and enhancing contrast. DAPI images can be subtly sharpened with a 7×7 Hi-Gauss high-pass spatial filter or by de-convolution to accentuate minor details and heterochromatin differentiation of the chromosomes.
4. The different FISH signals are merged in a multichannel mode (Fig. 1). Fluorescent images are displayed in grey or light blue

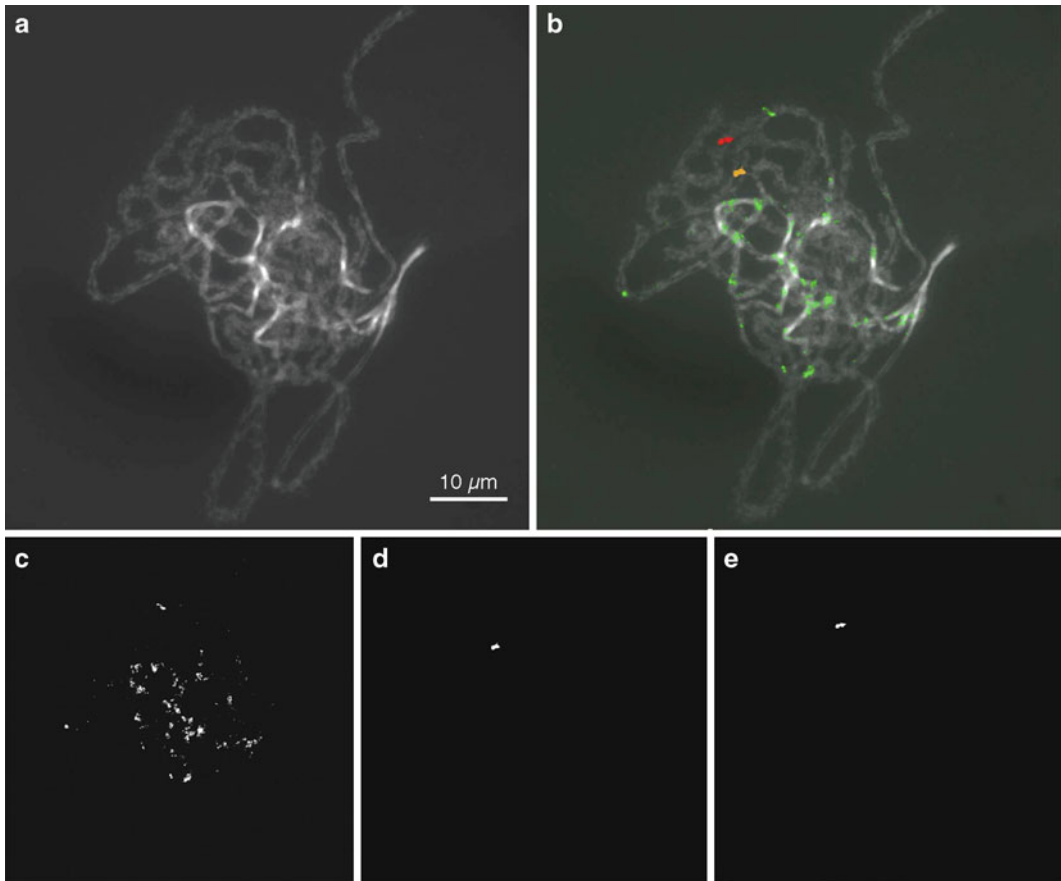


Fig. 1 Example of BAC-FISH painting on a pollen mother cell at pachytene of *Medicago truncatula*. (a) Well-spread pachytene chromosomes stained with DAPI; (b) three-color FISH with BACs labeled with the green fluorescing FITC (c), the orange fluorescing Cy3 (d), and the red fluorescing Cy3.5 (e), respectively. The individual BAC images were separately captured with a black/white camera and their images stacked in pseudo-colored spot channels using Adobe Photoshop software. Note that the green fluorescing BAC has multiple foci due to repetitive sequences in the BAC DNA

for DAPI and pseudo-colored for the other signals. The images can be processed in specialized image capturing software or in Adobe Photoshop CS. Open-source ImageJ image processing software can be used for measurements and for straightening the chromosomes using the plug-in of Kocsis et al. [20].

5. For chromosome lengths and gap sizes the images of several different pachytene spreads have to be measured. The comparison is also important to establish variation in the heterochromatin patterns.

4 Notes

1. The choice of Cot-DNA for blocking requires some experience. For small-genome plants (like *Arabidopsis*) with low amounts of repetitive sequences in the euchromatin it is in general not needed to prepare Cot-DNA for blocking, but larger genomes, especially with BACs containing retrotransposons regions need Cot-DNA. It is recommended first to isolate different Cot fractions (Cot-1 Cot10, Cot100, Cot500) and use their DNA as probe in a FISH experiment to see where most of the repetitive sequences reside. This information can then be used to decide which Cot fraction is the best for blocking.
2. The same basic protocol can be used for cross-species chromosome painting, using BAC probes on the chromosomes of related species. In such cases hybridization and washing stringencies may be lowered if donor DNA in the probe and recipient DNA in the chromosome slide are not highly homologous. To do so, the formamide concentration is lowered from 50 to 35 % or 20 % [21].

References

1. Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W et al (2010) Genome sequence of the palaeopolyploid soybean. *Nature* 463:178–183
2. Young ND, Debellé F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK et al (2011) The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* 480: 520–524
3. Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M et al (2008) Genome structure of the legume, *Lotus japonicus*. *DNA Res* 15:227–239
4. Varshney RK, Chen W, Li Y, Bharti AK, Saxena RK, Schlueter JA et al (2012) Draft genome sequence of pigeonpea (*Cajanus cajan*), an orphan legume crop of resource-poor farmers. *Nat Biotechnol* 30:83–89
5. Schubert I, Fransz PF, Fuchs J, de Jong JH (2001) Chromosome painting in plants. *Methods Cell Sci* 23:57–69
6. Sharma AK, Sharma A (2001) Chromosome painting—principles, strategies and scope. *Methods Cell Sci* 23:1–5
7. Kato A, Vega JM, Han F, Lamb JC, Birchler JA (2005) Advances in plant chromosome identification and cytogenetic techniques. *Curr Opin Plant Biol* 8:148–154
8. Jiang J, Gill BS (2006) Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. *Genome* 49:1057–1068

9. De Jong JH, Fransz PF, Zabel P (1999) High resolution FISH in plants—techniques and applications. *Trends Plant Sci* 4:258–263
10. Peterson DG, Schulze SR, Sciara EB, Lee SA, Bowers JE, Nagel A et al (2002) Integration of Cot analysis, DNA cloning, and high-throughput sequencing facilitates genome characterization and gene discovery. *Genome Res* 12:795–807
11. Yuan Y, San Miguel PJ, Bennetzen JL (2003) High-Cot sequence analysis of the maize genome. *Plant J* 34:249–255
12. Campell BR, Song YG, Posch TE, Cullis CA, Town CD (1992) Sequence and organization of 5s ribosomal RNA-encoding genes of *Arabidopsis thaliana*. *Gene* 112:225–228
13. Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal-Rna genes from wheat and barley. *Nucleic Acids Res* 7: 1869–1885
14. Richards EJ, Ausubel FM (1988) Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell* 53:127–136
15. Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol Biol Rep* 15:8–15
16. Kulikova O, Gualtieri G, Geurts R, Kim D-J, Cook D, Huguet T, de Jong JH, Fransz PF, Bisseling T (2001) Integration of the FISH pachytene and genetic maps of *Medicago truncatula*. *Plant J* 27:49–58
17. Woo SS, Jiang J, Gill BS, Paterson AH, Wing RA (1994) Construction and characterization of bacterial artificial chromosome library of *Sorghum bicolor*. *Nucleic Acids Res* 22: 4922–4931
18. Zwick MS, Hanson RE, Islam-Faridi MN, Stelly DM, Wing RA, Price HJ, McKnight TD (1997) A rapid procedure for the isolation of Cot-1 DNA from plants. *Genome* 40:138–142
19. Zhong XB, de Jong JH, Zabel P (1996) Preparation of tomato meiotic pachytene and mitotic metaphase chromosomes suitable for fluorescence in situ hybridization (FISH). *Chromosome Res* 4:24–28
20. Kocsis E, Trus BL, Steer CJ, Bisher ME, Steven AC (1991) Image averaging of flexible fibrous macromolecules: the clathrin triskelion has an elastic proximal segment. *J Struct Biol* 107: 6–14
21. Mandáková T, Lysak MA (2008) Chromosomal phylogeny and karyotype evolution in $x=7$ crucifer species (Brassicaceae). *Plant Cell* 20: 2559–2570

Legume Genomics

Methods and Protocols

Rose, R. (Ed.)

2013, XI, 316 p. 53 illus., 23 illus. in color., Hardcover

ISBN: 978-1-62703-612-2

A product of Humana Press