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RESEARCH ARTICLE

# Technological exploration of BAC-FISH on mitotic chromosomes of maize

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**Abstract** The rice BAC-DNA was used as probes and fluorescence *in situ* hybridization (FISH) was applied to the interphase and metaphase mitotic chromosomes of maize. To optimize the BAC-FISH technique, we respectively assayed the effect of several factors, including maize or rice genomic $C_o$t DNA used as blocking reagent of DNA, washing temperatures and FAD concentration in the washing buffer and in the hybrid solution. The results show that $C_o$t DNA of maize genome blocked the repetitive sequence of the rice BAC-DNA when the $C_o$t value was below 50. Meanwhile, it was necessary to adjust the $C_o$t value according to the different probes and their ratios. Decreasing the concentration of FAD in the hybridization mixtures, adjusting the washing rate after hybridization, and most especially, blocking the rice-specific repetitive sequences of BAC-DNA could improve the positive signals of BAC-FISH.

**Keywords** maize (*Zea mays* L.), BAC-FISH, Comparative cytogenetic mapping, $C_o$t DNA

## 1 Introduction

The accomplishment of the rice genome sequencing project, the fast advancement in research of rice genomics and functional genomics, the construction of the physical maps and heredity maps of rice with high resolution and precision (International Rice Genome Sequencing Project, 2005), as well as the close relationship and the evolutionary synteny and co-linearity among gramineous plants (Haberer et al., 2005) laid the foundation for comparative genomics study among gramineous plants bridged by the rice genome data.

Maize is an important economic crop and is also an important model plant for genetic research. However, its genome is large and contains more than 80% repetitive sequences (Clark et al., 2004) which add more difficulties to its genomic research. In molecular cell genetics, *in situ* hybridization (ISH) is widely used in the comparative genome mapping, construction of physical maps, the karyotype analysis, localization of important function genes and the examination of transgenic insertion. Thus, it has become one of the most important tools for the research of maize genomics and functional genomics, such as identification and karyotyping of maize chromosomes by locating the repetitive sequences on maize chromosomes by fluorescence *in situ* hybridization (FISH) (Chen et al., 2000). Although it is possible to localize the single copy gene on maize chromosomes, the sensitivity is very low (Wang and Chen, 2005). This disadvantage can be improved by employing bacterial artificial chromosome (BAC) clones as probes in some species (Jiang et al., 1995). However, the complexity of maize chromosomal structure and composition results in the interference of non-specific signals caused by repeated sequences in the BAC clones. As a result, the application of BAC-FISH on maize has been restricted and relevant reports are few.

Besides selecting the BAC clones with a small amount of repetitive sequences for the probe, we need to optimize the condition of the BAC-FISH system which can reduce or eliminate the influence of repetitive sequences of the probe. In the present study, five selected rice BAC clones were used as probes of BAC-FISH on the maize mitosis chromosomes and we examined the influencing factors on FISH hybridization specificity, including $C_o$t DNA value, hybridization condition and stringency of the eluting progress in order to optimize the system of rice BAC-FISH on maize mitosis chromosomes.

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## 2 Materials and methods

### 2.1 Plant materials

Mo17 maize inbred lines were used. Root tips were harvested when they were 0.5–1 cm from seedlings grown on moist filter papers in a culture tank (Ning et al., 2000). The excised root tips were pretreated in water-supersaturated α-bromonaphthalene in the dark at room temperature for 3 h and then fixed in 100% ethanol/acetic acid (3:1) (v/v) at 4°C overnight. Mitotic chromosome preparation was performed using the routine protoplast technique as described by Song et al. (1995).

### 2.2 BAC labeling

Five rice BAC clones: NR1, NR2, NR3, NR4 and NR5, were kindly provided by Professor Qifa ZHANG at the National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University. The insertion fragments of clones were 125–185 kb and were extracted with the standard alkaline lysis method (Sambrook et al., 1989). The purified DNA was labeled with the nick-translation kit (Sino-America Biotechnology Company, China). The reaction mixture was incubated at 15°C for 1.5 h and terminated by adding 1 μL 0.5 mol/L EDTA (pH 8.0). Labeled probe was separated from unincorporated nucleotides by passage through a Sepharose CL-6B (Sigma) column.

### 2.3 Genomic DNA extraction and $C_o$t DNA preparation

Maize and rice genomic DNAs were extracted with the CTAB method (Doyle and Doyle, 1990) and the genomic DNAs were purified. $C_o$t DNA was prepared as described by Zwick et al. (1997).

### 2.4 *In situ* hybridization and detection

*In situ* hybridization was performed according to the procedures described by Gustafson and Dille (1992). The hybridization mixture contained 50% deionized formamide, 10% sodium dextran sulphate, 2 × SSC, 1 mg/mL salmon sperm DNA, $C_o$t-1 DNA and 30 ng probes BAC DNA, and co-denatured at 90°C for 10 minutes. Hybridization was performed overnight at 37°C. Chromosomes were counterstained with 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, Burlingame, CA, USA). Chromosome preparations were examined with an Olympus BX-60 fluorescence microscope. Images were captured with a cooled CCD camera (1401E B0, Sensys, Photometrics) by using the Metamorph software (Universal Imaging, Version 4.6r5). The final images were adjusted with Adobe Photoshop.

## 3 Results

### 3.1 Blocking of probe repetitive sequence by $C_o$t DNA

There are large amounts of repeat sequences in maize genome. The specificity of hybridization signals will be greatly reduced when the probe has the homologous repetitive sequences of the maize genome. Therefore the homologous repetitive sequences of the rice BAC probe were blocked by $C_o$t-1 DNA, $C_o$t-50 DNA and $C_o$t-100 DNA from maize genome to eliminate the impact of the probe sequences homologous with repetitive sequences of maize genome and the unblocked probes were used as a control. The hybridization signals of corn NR6 BAC-DNA probe in the rice chromosome were found throughout the entire genome (Fig. 1A), which shows that Rice NR6 BAC-DNA contains homologous sequences with maize repetitive sequences

We used maize $C_o$t-1 DNA to block the homologous repetitive sequences (Probe: $C_o$t-1 DNA = 1:10), and biotin-labeled 45S rDNA for control (Fig. 1B and C). The hybridization results show two specific hybridization signals of 45S rDNA (red) and non-specific hybridization signal of Rice NR6 BAC. Using $C_o$t-50 DNA of maize genome as a block (Probe: $C_o$t-50 DNA = 1:5), FISH results show that the specific signal of the 45S rDNA probe still existed, but the signal of NR6 BAC-DNA was not found (Fig. 1D). Using the $C_o$t-100 DNA probe

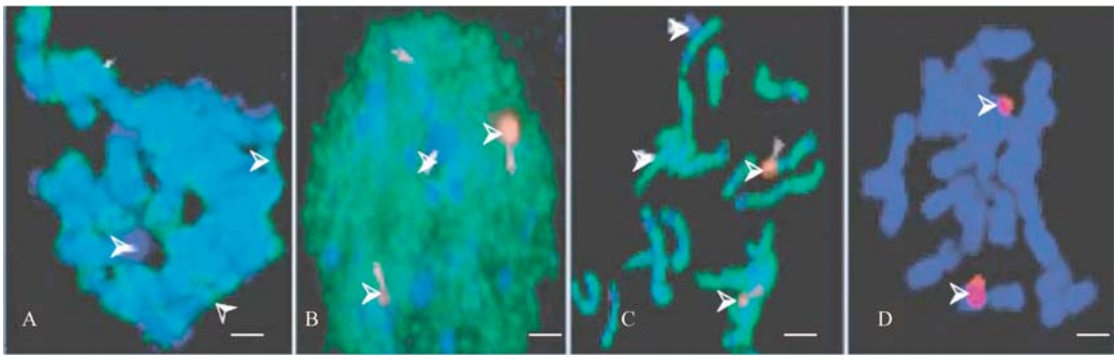


**Fig. 1** FISH signals with blocked $C_o$t DNA in maize chromosomes. Note: (A) CK; (B) and (C) $C_o$t-1 DNA from maize genetic DNA; (D) $C_o$t-50 DNA from maize genetic DNA. Green: signals of BAC-probes; Red: signals of 45S rDNA-probes, Bars = 10 μm.

to block the genomic DNA, the signal of the 45S rDNA could not be detected any more. These suggest that: (1) the maize genome $C_o$t-1 DNA does not contain the entire types of the repetitive sequences in NR6 BAC-DNA. (2) The homologous repetitive sequences between NR6 BAC-DNA and maize genome can be blocked by the $C_o$t-50 DNA of maize genome. The specific hybridization between NR6 BAC-DNA and target DNA sequence requires that the Cot value be reduced to 50.

### 3.2 Stringent conditions of eluting after hybridization

Given sufficient FISH signals, stringent eluting solution can reduce the effect of non-specific hybridization signal. Koumbaris and Bass (2003) used 50% formamide at 30°C in the hybridization of Sorghum BAC clone to the maize chromosome. Zhang et al. (2004) used 50% formamide at 42°C in the BAC-FISH of wheat. No fluorescent hybridization signal of mixed probe of NR6 BAC-DNA (Digoxin) and 45S rDNA (Biotin) was detected when the washing was done with 20% formamide for 10 min at 42°C. The signals of other rice's BAC DNA (NR2, NR3, NR4, NR5 and NR7) were not detected in the same solution either, even when the hybridization time was extended to 3 days (Yoshido et al., 2005). When the washing temperature was 37°C, the hybridization signal of rice NR6 BAC-DNA was detected in maize metaphase chromosomes (Fig. 2). These results indicate that the washing temperature is one of the key factors for the BAC-FISH.

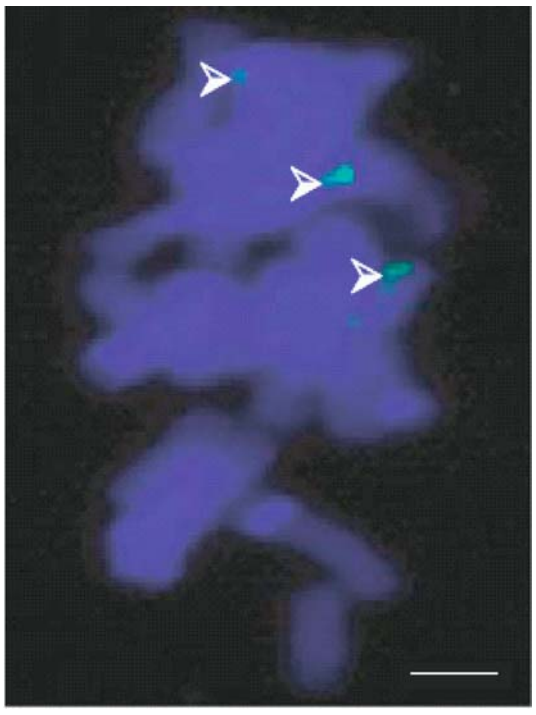

**Fig. 2** FISH signals with washing with 20% formamide at 37°C. Note: Green: signals of BAC-probes; Bars = 10 μm.

### 3.3 FAD concentration, blocking reagent and BAC-FISH

As the blocking reagent, BAC-DNA repetitive sequences can improve the efficiency of BAC-FISH. Thus, we prepared rice NR6 BAC $C_o$t-1 DNA mixed with NR6 BAC-DNA at 1:1 and added it into the hybridization solution containing 30% FAD, 10% sulphuric acid glucose, 20 × SSC, 1 μg ssDNA and 1 μg probes BAC DNA, and the eluting procedures were conducted as described above. Three specific hybridization signals were shown with a high consistency both in the interphase nucleus (Fig. 3A) and on the metaphase chromosome (Fig. 3B). It suggests that using NR6 BAC DNA repetitive sequences, as a blocking reagent, is conducive to improve the FISH hybridization specificity on maize mitotic chromosomes.

Based on the above results, the rice NR6 BAC-DNA FISH probe hybridization conditions can be summarized as follows: (1)The hybridization solution contains 30% FAD, 10% sulphuric acid glucose, 20 × SSC and 1 μg ssDNA. (2)After hybridization, eluting solutions use 20% formamide solution with the temperature below 37°C. (3)Using rice NR6 BAC $C_o$t-1 DNA as a blocking reagent can effectively reduce or eliminate the background of hybridization signals.

According to the hybridization condition of rice NR6 BAC-DNA on maize mitotic chromosome, we performed FISH on maize mitotic chromosome with rice NR2, NR3, NR4 and NR7 BAC-DNA. The hybridization results show that rice NR2 BAC-DNA probe displayed a very clear hybridization signal (Fig. 4A). NR3 BAC-DNA probe displayed clear hybridization signals in the interphase nucleus (Fig. 4B) and on the metaphase chromosomes (Fig. 4C) and NR4 and NR7 BAC probes also show similar results (Fig. 4D–G). These demonstrate that the improved BAC-FISH hybridization conditions are suitable for hybridization of rice BAC clone probe with maize mitotic chromosomes and are effective on reducing the background interference of probe repetitive sequences.

## 4 Discussion

The Gramineous crop genomes are highly conserved. Regarding their molecular markers, genome composition and structure, and DNA sequence, the genes among different species within the Gramineous family share widespread synteny and co-linearity. Maize and rice belong to the Gramineous plants (Haberer et al., 2005). They have a common ancestor, a close relationship and a high homology in DNA level. This provides a possibility to localize the rice BAC clone on the maize chromosomes and to localize, reveal and clone the maize genes based on the corresponding known rice genes (Ren et al., 1997). The

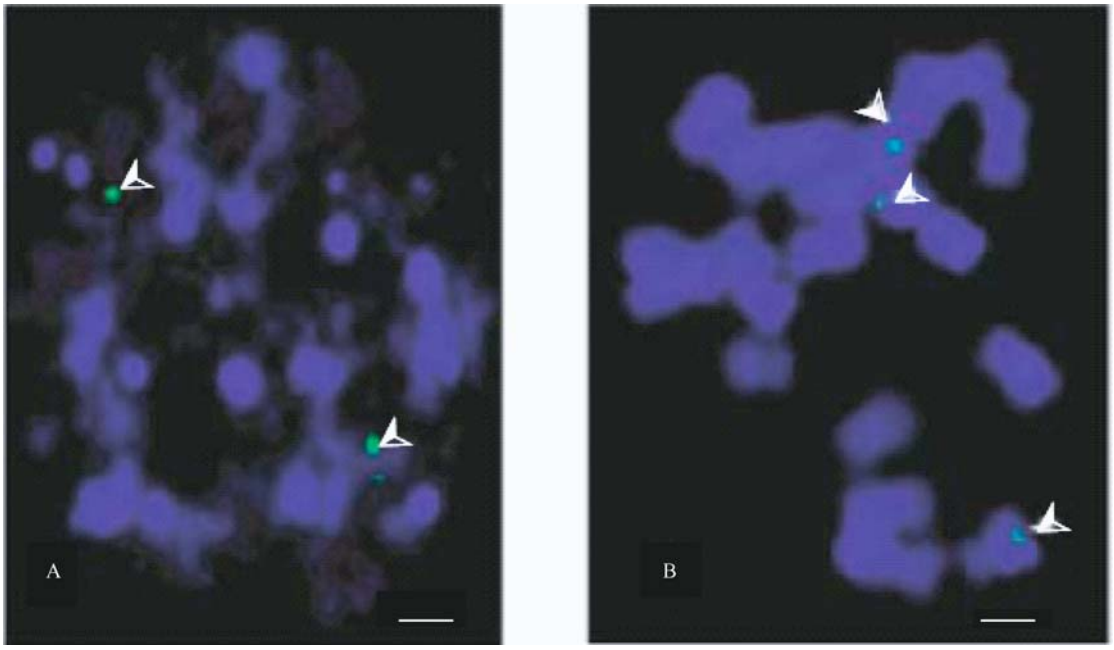


**Fig. 3** FISH signals with blocked $C_o$t-1 DNA from NR6 BAC DNA of rice. Note: (A) Interphase nucleus chromosomes; (B) Mitotic metaphase chromosomes. Green: signals of BAC-probes; Bars = 10 μm.

advanced BAC-FISH technology is the prerequisite to achieve this goal.

However, there are many rearrangements in the evolution of maize genome and 70% of the genome are the repetitive sequences, especially retrotransposons (Lai et al., 2004). The FISH signals of rice BAC probes on maize chromosome increase with the copy number of the repeats. Genome rearrangement will also interfere with FISH signals. One way to solve this problem is to use the repetitive sequences to block the probe sequences

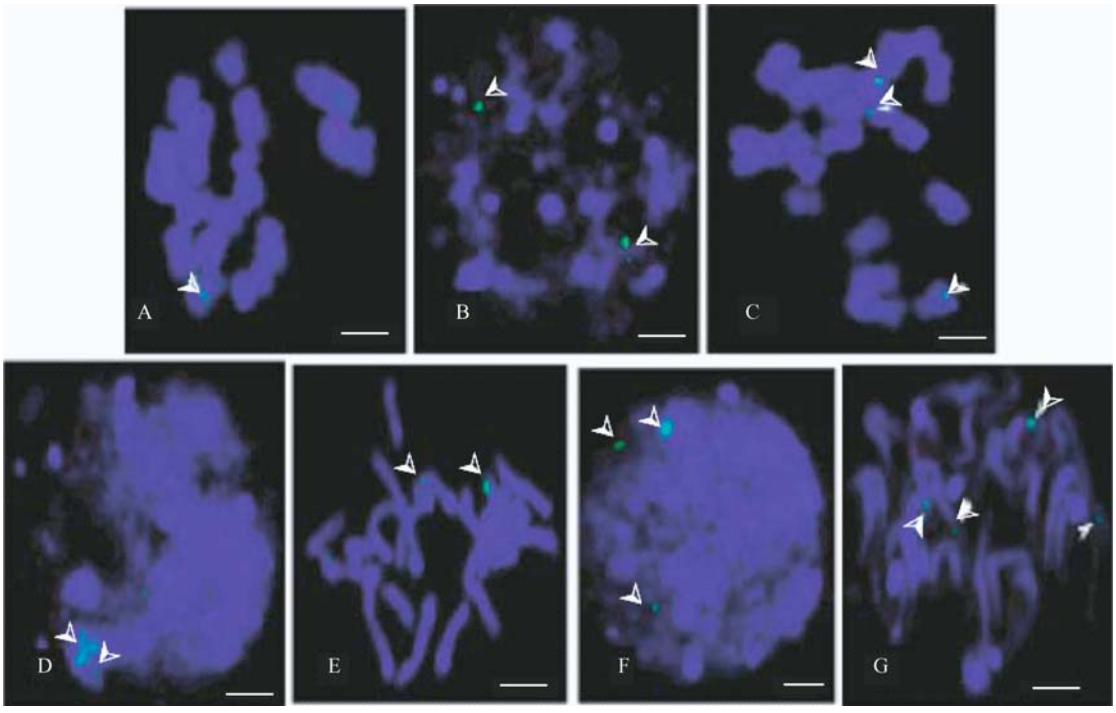


**Fig. 4** FISH signals from other rice's BAC-probes. Note: (A) NR2; (B) and (C) NR3; (D) and (E) NR4; (F) and (G) NR7; Thereinto (B), (D) and (F) Interphase nuclear chromosomes; (C), (E) and (G) Mitotic metaphase chromosomes. Green: signals of BAC-probes, Bars = 10 μm.

with homology. After blocking of NR6 BAC with maize genome $C_o$t-1, $C_o$t-50 and $C_o$t-100 DNA respectively, we found $C_o$t-1 DNA was insufficient to block the homologous sequence in NR6 BAC, while $C_o$t-50 DNA contained all NR6 BAC probe sequences except the sequence whose copy number was lower than that of 45S rDNA. It indicates that the copy number of the repetitive sequence of NR6 BAC is more than that of 45S rDNA sequence in maize genome. We also found there were sequences which were homologous with maize retrotransposons and Mutator's transposase in NR6 BAC. As retrotransposons are highly repetitive in maize genome and Mutator are also repeated, they possess the priority in the process of renaturation. $C_o$t-50 DNA harbored sequences homologous with maize retrotransposons and Mutator's transposase and it did not contain sequences similar to 45S rDNA. When blocked with $C_o$t-50 DNA, the homologous sequences in NR6 BAC preferred to bind $C_o$t-50 DNA. Therefore, $C_o$t-50 DNA blocked the NR6 BAC hybridization signal, while it did not block 45S rDNA hybridization signal.

The successful application of DOP-PCR technology in BAC-FISH provides an idea of changing the probe from the low copy sequences to repeated sequences (Wienberg et al., 1997*). Therefore, we prepared $C_o$t DNA with NR6 BAC-DNA. Certainly, the $C_o$t DNA which contains maize retrotransposon sequences will block maize chromosome retrotransposons sequence, thus, enhancing the specificity of hybridization signals. In addition, we can also improve the specificity of hybridization signals by increasing FAD concentration or stringency of eluting condition (McConaughy et al., 1969).

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