BREEDING AND GENETICS

Development of an Improved Method of Mitotic Metaphase Chromosome Preparation Compatible for Fluorescence in situ Hybridization in Cotton

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ABSTRACT

Molecular cytogenetic techniques, especially Fluorescence In situ Hybridization (FISH) and Genomic In situ Hybridization (GISH), are excellent tools to study the structure and function of chromosomes, polyploidy, aneuploidy, alien gene introgression and genome evolution and physical mapping of genes. For many applications, in situ hybridizations require reliable and efficient methods of chromosome preparation with well preserved and dispersed chromosomes and little or no cell wall debris. However, such protocols have not yet been published for cotton mitotic chromosome preparations. In the current study, an improved method for preparing mitotic metaphase chromosomes of tetraploid cotton was developed. Root tips were collected from lab-grown three-day-old cotton seedlings and pre-treated with high-pressure nitrous oxide (N_2O). Following fixation with acetic acid, root tip meristems were removed, enzymatically digested, gently macerated, and then processed to create suspensions of protoplasts in acetic acid and methanol. Suspended protoplasts were then dropped onto a glass slide in order to distribute the protoplasts and spread the chromosomes. Using this approach, acceptable mitotic indexes and high-quality chromosomal spreads with no cell wall debris were obtained. This drop method of preparing chromosome spreads was tested for its compatibility for in situ hybridization using rDNA as probe in FISH. Additionally, varying pre-treatment times with N_2O were investigated. Our results show that the combination of a 95-minute N_2O pre-treatment, a 55-minute enzymatic degradation, and the "drop method" efficiently produce cotton mitotic metaphase preparations. This new method will expectedly improve the efficiency and efficacy of GISH, FISH, and single-copy multicolor FISH, which will have a multitude of ramifications in cotton genetics and breeding.

Cytogenetic analysis and chromosome manipulation are essential to genetic and genomics analysis, as well as germplasm introgression. Molecular cytogenetic techniques, especially fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH), are excellent tools to study the structure and function of genomes, chromosome land marks, distribution of recombination across the chromosomes, polyploidy, aneuploidy, alien gene introgression and genome evolution. However, efficiency and success of these capabilities depends upon good chromosome preparations. Higher mitotic indexes and well-spread or dispersed chromosomes whose morphology was well preserved during the preparation process are an essential requirement for metaphase chromosome spreads to be used in FISH and GISH experiments. Efficient chromosomal spreads with less contamination and/or hindrance from cell wall debris are even more important for performing FISH with single copy probes in plants. This is especially true in cotton due to the copious and diminutive disposition of the chromosomes, which plagues the identification of physical landmarks such as centromeric constrictions and the discrimination of sister chromatids in mitotic metaphase cells. Besides having numerous small chromosomes, it has been observed that cotton is a particularly troublesome species in regard to chromosome aggregation (Halfmann et al., 2007). Furthermore, due to its dicotyledonous nature, cotton has only the main taproot available for mitotic chromosomal studies. In the absence of efficient chromosome preparation this often becomes a limiting factor for analyzing single plants using FISH and GISH techniques, especially when chromosome morphology and cytological landmarks are pivotal

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for such analysis. To date, the majority of cotton cytogenetic work has utilized the squash method to spread chromosomes (Crane et al., 1993; Hanson et al., 1996; Peng et al., 2012; Wang et al., 2006). The squash technique, which involves the placement of a cover slip over a dissected root tip or previously digested cells to flatten and spread the chromosomes, can cause damage and loss of chromosomes and requires a high degree of technical skill. Therefore, there is a critical need to develop a protocol that could increase the mitotic index and improve the efficiency of cell synchronization and chromosomal spreads, while limiting contamination from cell wall debris in the preparations used for in situ hybridizations.

Several methods have been used to increase cell synchronization and mitotic index in plants such as ice water, hydroxyurea (HU), mimosine, aphidicolin, and nutrient deprivation (Arumuganathan et al., 1991; Halfmann et al., 2007; Pan et al., 1993). Historically, compounds such as colchicine and amiprophos-methyl (APM) are then used to arrest root cells at metaphase (Halfmann et al., 2007; Hanson et al., 1996). Systematic analyses in cotton were done by Halfmann et al. (2007). They investigated procedures for cell synchronization of root tips using hydroxyurea as well as the efficacy of several known chemicals for metaphase accumulation. Anti-tubulin compounds provided average metaphase indices of 0.3 or higher on synchronized root tips and were preferable to colchicine (Halfmann et al., 2007). In animals, cells have long been treated with a hypotonic solution to improve the spread of chromosomes through swelling and bursting of the cells (Hsu and Pomerat, 1953). However, this approach is not as effective in plants since the presence of the cell wall inhibits lysis. Halfmann et al. (2007), noted that nitrous oxide has the highest dispersing effect in cotton, though the mitotic index observed in these spreads was less than expected using the traditional squash method of chromosome preparation. However, the best chromosome spreads were achieved when a nitrous oxide treatment was combined with the drop method in corn and wheat (Danilova et al., 2012; Kato, 1999). Procedures for the isolation of meiotic pachytene and interphase chromosomes in cotton have been described by Peng et al. (2012) but a protocol which can yield efficient mitotic metaphase chromosome preparations is not available in cotton.

Two methods are used for multicolor FISH in plants, indirect and direct methods. The indirect method mostly uses biotin and digoxigenin as labels. In the next step, reporter molecules are used to detect the label in the probe, avidin and streptavidin for biotin and anti-digoxigenin antibodies for digoxigenin. These reporter molecules are conjugated with a signal generating system, which makes the visualization of the probe possible. Most of the cotton FISH experiments used indirect FISH to visualize gene clusters and repeats (Mukai et al., 1993; Pederson and Langridge, 1997; Ji et al., 1997, 1999; Turnbull et al., 2003; Wang et al., 2007, 2010) which is time and resource consuming. However direct FISH, where the label is incorporated directly into the nucleic acid probe through nick translation, has been found to be efficient for single copy FISH even in repeat rich genomes (Danilova et al., 2012; Kato et al., 2004, 2006; Wang et al., 2007). An improved chromosome preparation with dispersed chromosomes and minimal or no cell wall debris could eliminate the requirement for signal amplification in FISH in cotton.

In an attempt to develop a reliable, and effective way to prepare cotton mitotic chromosomes for FISH and related cytogenetic techniques, the objectives of the current study were to: i) to test the feasibility of mitotic metaphase chromosome preparation in cotton using root tip nitrous oxide treatment and drop method of spreading, ii) test varying treatment times of nitrous oxide in order to find the optimal duration of nitrous oxide exposure in cotton, and iii) evaluate this new method of preparing chromosome spreads for its compatibility to in situ hybridization using wheat rDNA as a probe in direct FISH.

MATERIALS AND METHODS

Chromosome preparation. A modified version of the nitrous oxide and air drying method demonstrated in maize by Kato (1999) was used to prepare cotton mitotic metaphase chromosomes for fluorescent in situ hybridization. Seeds of the *Gossypium hirsutum* L. variety 'TM-1' (NC2630, PI 607172 originally obtained from Josh Lee) were germinated in moist vermiculite within plastic germination boxes (Figure 1). The seeds were kept in the dark at 30°C in a digital laboratory incubator (VWR International, Radnor, PA) for two to three days.
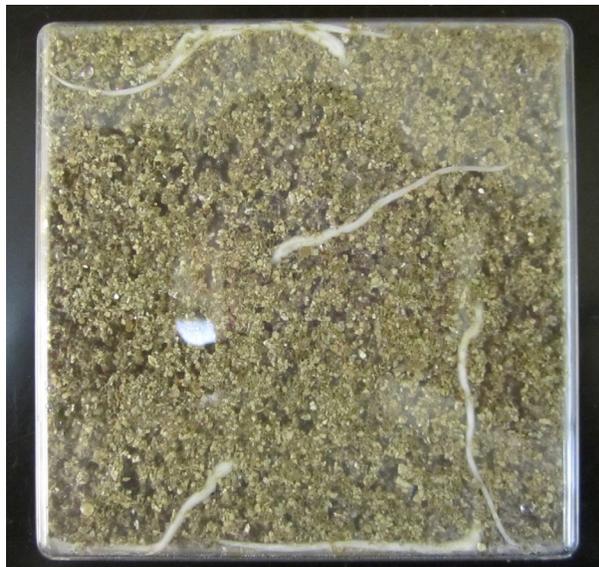
Figure 1: Image of the bottom of the plastic box two days after planting seeds in moist vermiculite showing the size of roots that were harvested for mitotic metaphase chromosome preparations.

Prior to root tip removal, standard 1.5mL Eppendorf micro-centrifuge tubes were prepared for sample collection. Three to four holes were placed in the top of each tube with a dissecting needle. From healthy and vigorous seedlings (Figure 1), 2-4cm long root tips were removed and placed in the micro-centrifuge tubes. One to two roots were placed in each tube and the tubes were misted lightly with double distilled water using a standard spray bottle.

The 1.5mL tubes were placed in a custom-built gas-pressure chamber (Figure 2A, B) manufactured by the Murr Instrument Shop, University of Missouri-Columbia. Nitrous oxide was applied at a pressure of 160psi (~10.9 atm) for 1 hour and 45 minutes using a custom-built apparatus (Figure 2A, B). To test the effect of length of N_2O exposure time on chromosome condensation, times of 80, 95, and 120 minutes were also used.
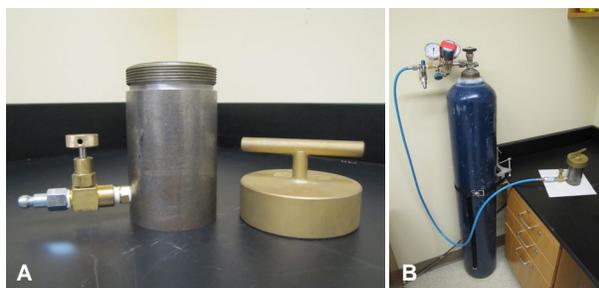
Figure 2A, B: Custom-built gas-pressure chamber for exposing root tips to nitrous oxide (N_2O). A) Side view of the pressure chamber in which root tips are treated. B) The entire N_2O delivery system.

Cells in the root tips were then fixed by filling each tube with 90% ice-cold acetic acid and incubating on ice for 15 minutes. Following fixation, root tips were transferred to fresh tubes containing ice-cold 70% ethanol. The solution was gently mixed using a glass pipette; ethanol was removed, and then replaced with fresh ice-cold 70% ethanol. This was repeated 3 times to remove the acetic acid residue. Following the final removal of ethanol, ice-cold 1x citric buffer was added. Samples were mixed gently using the glass pipette and citric buffer was constantly removed and replaced with fresh buffer for 10 minutes.

Following the last removal of citric buffer, the root tips were cleaned on filter paper. A one to two mm long section of the milky white region was removed and transferred to a standard 0.7mL Eppendorf tube containing 20µL of ice-cold enzyme solution (1% pectolyase Y-23 (MP Biochemicals, Solon, OH), 4% cellulase Onozuka R-10 (Yakult Pharmaceuticals, Tokyo, Japan) in 1x citric buffer). The sample tubes were then placed in a Big Shot III Hybridization Oven (Boekel Scientific, Feasterville, PA) for incubation at 37ºC for 55 minutes.

Immediately following digestion, tubes were plunged into ice. The digested root tips were then washed with ice-cold 1x TE buffer, followed by 2-3 washes in ice cold 100% ethanol using a standard glass pipette. After thoroughly removing the ethanol, 30µL of freshly prepared 75% acetic acid – 25% methanol was added. The remains of the root tips, sufficiently weakened by the enzymatic degradation, were then ground gently using a rounded dissecting needle.

Pre-cleaned Gold Seal microscope slides (Thermo Fisher Scientific Inc., Houston, TX) were labeled and kept on a Plexiglas scaffold. Using a standard pipette, 8 µL of the cell suspension was dropped onto the marked spot on the slide. The slides were then allowed to dry by placing them inside a homemade humidity chamber for three to five minutes. After drying, slides were viewed under a phase contrast Leica DME microscope (Leica Microsystems, Wetzlar, Germany) in order to ensure selection of slides with good chromosomal spread for hybridization. Chosen slides where then placed on a thin piece of cardboard and cross-linked on a SpectroLinker XL-1000 UV Crosslinker (Spectronics Corporation, Lincoln, NE). Slides were then stored overnight at -20°C in a standard microscope slide box.

Fluorescence in situ hybridization. The DNA insert in clone PTA71 was used as a probe in the FISH experiment. Clone PTA71, the 18S-28S rDNA, is a 9-kb *Eco*RI fragment from common wheat (*Triticum aestivum*) (Gerlach and Bedbrook, 1979). It contains the coding sequence for the 18S, 5.8S, and 25S rDNA genes and the intergenic spacer sequences (Gerlach and Bedbrook, 1979). Plasmid PTA71-containing *E. coli* bacteria were grown in 3 mL of LB broth containing ampicillin in an incubating shaker. Plasmid DNA was isolated using a QIAGEN plasmid mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microgram of plasmid DNA was labeled with fluorescein-12-dUTP (Enzo Life Sciences Inc., Farmingdale, NY) using nick translation according to the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN).

Five μL of denatured salmon sperm DNA (140ng/μL in 2x SSC) were dropped onto the center of each cell spread and covered with a 22x22mm cover slip. Root-tip DNA (on slide) and probe DNA (in 200μL thin-wall PCR tubes) were denatured by floating in the boiling water baths for five minutes at 100°C. The water bath consisted of a small aluminum tray covered with a wet (DI water) layer of Kimwipes®. Slides and tubes were pressed firmly onto the Kimwipes and covered with a plastic pipette tip box lid. The entire assembly floated in a larger tray containing boiling water on a hot plate.

After denaturation, probes were plunged directly into ice. Denatured slides were placed on a thin metal sheet that had been placed upon the ice. The tube containing the probe was spun briefly to collect the total volume. The plastic cover slip was removed and 5 μL of the probe solution (40ng of labeled probe/μL) was placed onto the center of the slide. After addition of the probe, the slides were incubated in a humid storage container lined with Kimwipes soaked with 2x saline-sodium citrate (SSC) and placed in the Big Shot III Hybridization Oven at 37°C overnight.

The slides were then washed in 2x SSC at room temperature for five minutes in a Coplin jar. They were then washed at 42°C in 2x SSC for 20 minutes in an incubating shaker (Precision Reciprocal Shaking Bath, Thermo Fisher Scientific, Waltham, MA). After removing the slides from the bath, the bottoms and sides were briefly dried to remove excess SSC without allowing the tops to dry. One drop of ice-cold propidium iodide (PI) pre-mixed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) was added to each slide in order to stain the chromosomes. Slides

were covered with 24x50mm cover slips prior to visualization. Cells were examined under an Olympus BX53 Dark Fluorescence microscope (Olympus Corporation, Tokyo, Japan) connected to a Prior Lumen 200 light source (Prior Scientific, Cambridge, UK). Images were captured using a Hamamatsu ORCA-03 camera (Hamamatsu Photonics, Hamamatsu, Japan) and processed using cell sense dimension imaging software (Olympus Corporation, Tokyo, Japan).

C-banding. Chromosome preparations from root tips treated with N_2O for two hours were used for Giemsa staining in cotton. Chromosome preparations that were cross-linked were stored in -80°C and dehydrated in ethanol for five minutes. C-banding was done using a 10% solution of Giemsa stain (Gallard-Schlesinger Industries, Inc., NY) according to Gill et al. (1991), and visualized with a Zeiss photomicroscope III and photographed using a DP71 digital camera (Olympus Corporation, Tokyo, Japan). Images were captured in TIFF format at 432 dpi resolution.

RESULTS

Chromosome preparations. The mitotic metaphase chromosome preparation used here generated high-quality chromosomal spreads and proved highly adept at not only synchronizing and arresting cells in metaphase but also dispersing chromosomes in the cell. On each slide prepared, a large number of cells could be seen arrested in metaphase. In addition, many of them had sufficient spreads to allow the chromosomes to be easily counted and differentiated from one another. In Figures 3 and 5, all 52 chromosomes of the tetraploid cotton inbred line TM-1 are easily visible and distinguishable.

Following two-hour nitrous oxide treatment, cotton chromosomes were highly condensed and spread well (Figure 5). Because nitrous oxide is known to produce more condensed chromosomes than other arresting agents an attempt was made to offset the extreme condensation, by using N_2O treatment times of 80, 95, and 120 minutes.. The hope was to maintain the level of metaphase arrest and good chromosomal spread seen in the 110-minute treatment but generate longer, less condensed chromosomes to improve FISH sensitivity. The shorter N_2O treatments resulted in chromosomes that were slightly longer and less condensed than the 120-minute treatment, such that the sister chromatids and the narrowing at the centromere are more pronounced. However, chromosome clumping and a failure to generate good spreads became a

problem with the shorter exposures. This was especially true of the 80-minute treatment in which it was difficult to find cells where all 52 chromosomes could be unambiguously differentiated (data not shown). Clumping was less of a concern with the 95-minute treatment time as more cells with ideal spreads were found than with the 80-minute treatment. However, the number of spreads produced by the 95-minute treatment was slightly lower than that seen with the 120-minute treatment. Overall N_2O pre-treatment for 95 minutes at 160psi gave the best mitotic chromosome spreads in cotton (Figure 4A, B).

C-banding. Chromosome spreads were used in Giemsa staining to see if the drop method of chromosome preparation can help identify the C-banding pattern of cotton chromosomes. No C-banding pattern was observed in cotton except a homogeneous mild staining of all the chromosomes permitting the counting of the chromosome complement in a cell (Figure 5). This suggests that developing the C-banding pattern in cotton does not depend on the type of chromosome preparation used and supports previous observations that cotton does not show distinct C-banding pattern. Hence, no further efforts were made to test Giemsa staining on cotton chromosome preparations from variable N_2O treatments.

Figure 4A, B: Mitotic metaphase chromosomes of the *G. hirsutum* inbred line TM-1 with a 95-minute exposure to nitrous oxide. Chromosomes were stained with propidium iodide (PI) and therefore fluoresce red.
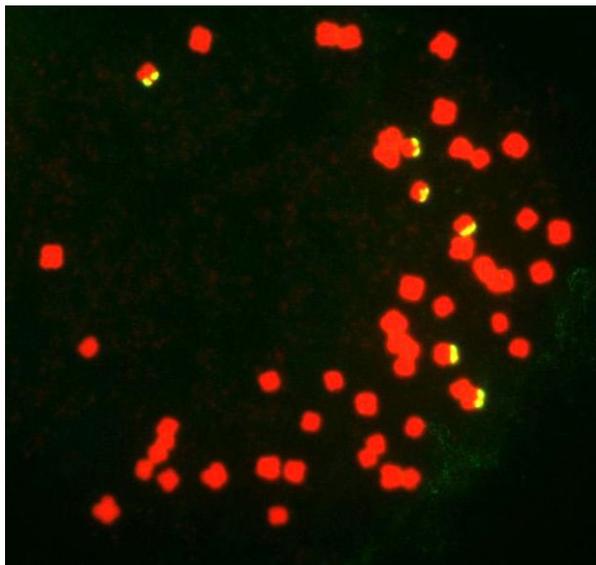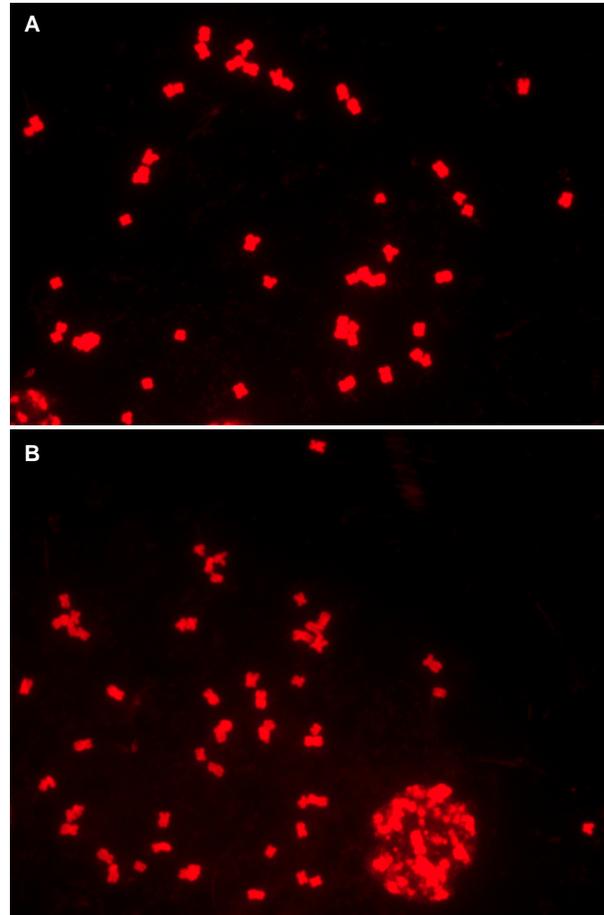

Figure 3: FISH on cotton mitotic metaphase chromosomes. All 52 chromosomes of *G. hirsutum* are visible and chromosomes were counterstained with propidium iodide (PI) and fluoresce red. FISH pattern of 18S-5S-26S rDNA tandem repeat probe (clone PTA71) visualized by yellow green fluorescein isothiocyanate (FITC) fluorescence. Six signals are visible corresponding to the three major rDNA loci in cotton.
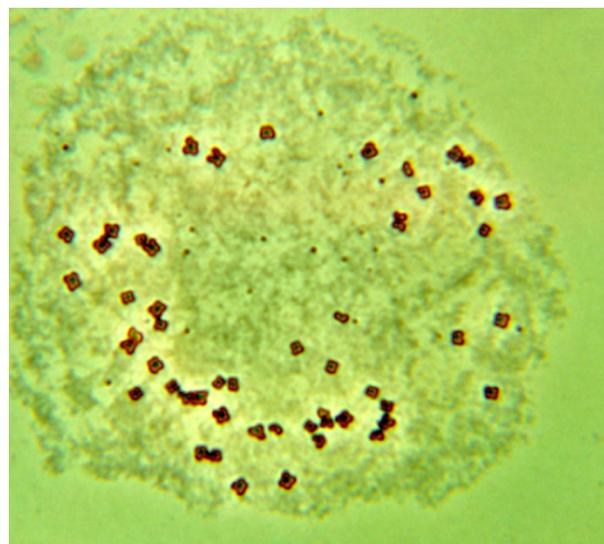

Figure 5: Giemsa stained cotton chromosome spreads prepared using the drop method. Chromosome preparations were from root tips that were treated with N_2O for two hours.

Fluorescence in situ hybridization. FISH with the wheat 18S-5S-26S rDNA tandem repeat probe showed excellent fluorescent signals on cotton chromosomes. Six different bands were detected, representing three different genomic loci, each belonging to a different pair of homologous chromosomes (Figure 3). Additionally, very little background fluorescence was detected, indicating that the hybridization protocol and probe worked efficiently in the present study. This further suggests that the drop method is compatible for obtaining good FISH signals and that the direct labeling of probes using fluorophores is feasible in cotton.

DISCUSSION

Here we report an efficient approach for generating mitotic metaphase chromosome preparations in cotton through the drop method combined with nitrous oxide pre-treatment and enzymatic digestion of root tips. Compatibility of this new method with FISH is demonstrated by using wheat rDNA as a probe in FISH experiments in cotton. As the rDNA sequences are highly conserved in plants (Gottlob-McHugh et al., 1990; Heslop-Harrison , 2000; Liu et al., 2005) use of rDNA sequences of wheat in this approach is sufficed.

Protocols for the preparation of pachytene and mitotic interphase chromosomes are developed in cotton (Peng et al. 2012). However, clumping, excessive condensation, high chromosome number and over- or insensitivity to many common arresting agents makes cotton a troublesome species for developing mitotic metaphase spreads. The findings of the study mentioned above (Peng et al., 2012) highlight many of the components necessary in a cotton mitotic metaphase preparation such as: a reliable method of isolating desired cell types, efficient digestion of cells, effective clearance of cellular debris, and obtaining an acceptable chromosomal spread. Our method addresses these concerns by using an enzyme digestion directly onto isolated root tip meristematic cells while using the drop method to clear debris and spread the chromosomes along with a nitrous oxide pre-treatment.

The use of nitrous oxide (N_2O) to produce large quantities of ideal mitotic metaphase chromosome preparations was first shown in maize (Kato, 1999). The superiority of the N_2O treatment comes not from an increase in the number of cells arrested, but rather from a pronounced positive effect on the dispersal of the chromosomes in the cell (Halfmann et al., 2007; Kato, 1999). This is in contrast to most other chemicals with metaphase arresting characteristics such as colchicine and APM, which tend to cause a cytologically undesirable clumping of the chromosomes (Halfmann et al., 2007; Kato, 1999). Since N_2O is applied as a high pressure gas, exposed cells subsequently bulge or swell in a manner similar to that of a hypotonic solution used in animal cytology studies. This helps to spread the chromosomes after the cell walls are macerated by enzyme digestion. The drop method, as opposed to the squash or smear method also facilitates chromosomal spread and helps to remove cell components and other debris left over from digestion. The large numbers of analyzable cells seen with this approach indicate that the drop method may be superior to both the squash method and the on-slide maceration approach for obtaining slides suitable for FISH in cotton.

Halfmann et al. (2007) found that while generating spreads of great quality, N_2O failed to arrest enough cells at metaphase in cotton to warrant its use in chromosome preparation. Nitrous oxide might be toxic in cotton, resulting in arrest of the cell cycle before the metaphase stage (Halfmann et al., 2007). However, our results show that there are still more than enough metaphase cells for cytogenetic work, especially for in situ hybridizations. Metaphase indexes were not calculated and N_2O was not compared to other chemicals in this study, but there is enough evidence to recommend the use of N_2O in the preparation of cotton mitotic metaphase chromosomes. Furthermore, the better spreads provided by N_2O compared to other chemicals, appears to be more than enough to offset any potential decrease in the mitotic index. Even if fewer cells are arrested in metaphase by N_2O, more cells are available for cytogenetic work due to this pronounced spreading effect and could be that the enzyme digestion method (drop method) allowed all the available protoplasts to be mounted on the slide. The exact reason why N_2O results in greater chromosomal spread is not definitively known. Unlike most mitotic arrestors such as colchicine, N_2O has no effect on the formation of the spindle fibers (Brinkley and Rao, 1973). Instead, it prevents the chromosomes from properly aligning along the metaphase plate, which in turn prevents the spindle fibers from attaching to the chromosomes (Brinkley and Rao, 1973). However, N_2O was routinely used

in chromosome doubling of haploids in wheat (Stan Cox, personal communication) indicating N_2O affects cell synchronization and chromosome segregation through an unknown mechanism.

When fully condensed, chromosomes of cotton are both small and relatively featureless aside from length and centromere location, rendering them less than ideal for many cytogenetic applications. N_2O tends to have a pronounced positive effect on chromosome condensation, which can exacerbate this limitation. Here, a shortened exposure to N_2O seemed to increase chromosome length and led to a decrease in condensation of mitotic metaphase chromosomes. This has the potential to improve the sensitivity and resolution of the N_2O pre-treatment for FISH in cotton. However, too short of an exposure to N_2O was found to increase clumping of chromosomes and impair spreading, negating one of the primary benefits of this approach. Therefore, a N_2O treatment time of 95 minutes at room temperature is recommended to produce the best mitotic metaphase chromosomes for FISH in cotton. This time provides a good balance of chromosome length and reduced condensation with an acceptable chromosomal spread.

The results of our FISH work are in agreement with the findings of Hanson et al. (1996) regarding the copy number of the 18S-5S-26S rDNA tandem repeat in the upland cotton (*G. hirsutum*) genome. Although Hanson et al. (1996) proposed that there are at least eleven 18S-5S-26S rDNA loci in cotton, only three of them were considered to be major loci. Of the remaining eight, one was considered intermediate and the other seven were deemed minor loci (Hanson et al., 1996). Results from Crane et al. (1993) and Ji et al. (1999) confirm the existence of minor and major rDNA loci in cotton. Most of these rDNA loci were also mapped to individual chromosomes using meiotic FISH (Crane et al., 1993; Ji et al., 1999). Our results showed six hybridization signals indicating three loci with pairs of signals coming from the same locus on homologous chromosomes (Figure 3). These three loci are likely the major loci detected by Hanson et al. (1996). Only the major loci may have been detected due to the use of a probe from an unrelated species. Nevertheless, our results show a novel and highly efficient method for preparing cotton mitotic metaphase chromosomes that is appropriate for FISH and in some instances, for chromosome counting.

Finally, as there are numerous developments in cytogenetics in recent times (Jiang and Gill, 2006; Kato et al., 2005; Peng et al., 2012; Wang et al., 2008) the improved chromosome preparation method reported in the present study will help describe the fine details of chromosome structure and behavior in cotton. As more genomes of cotton become sequenced, tools to study chromosomal organization and behavior will play a greater role in elucidating the structure and function of those genomes.

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