IMPROVED CELL CYCLE SYNCHRONIZATION AND CHROMOSOME DOUBLING METHODS IN COTTON

A Senior Honors Thesis

by

RANDAL HALFMANN

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2004

Major: Genetics

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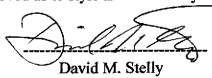

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April 2004

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ABSTRACT

Improved Cell Cycle Synchronization and

Chromosome Doubling Methods in Cotton. (April 2004)

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Cotton is an economically important crop in the U.S., but is relatively poorly characterized genetically. Unlike other, more researched crops, it is lacking highly effective methods for consistent cell cycle manipulation and chromosome doubling. Control over these biological features will enhance the ability to produce high quality cytological preparations of chromosomes and to double the number of chromosomes, i.e., induce polyploidy. The ability to routinely produce large numbers of high quality chromosome preparations from plants facilitates genomic characterization, while chromosome doubling allows integration of desirable genetic diversity from closely related plant species. This research establishes a procedure for cell cycle synchronization of root tips using hydroxyurea, and analyzes the efficacy of known chemicals for metaphase accumulation. Possibilities for adapting the procedure for improved chromosome doubling in shoot apical meristems are also introduced. Experimental evaluations were made by hydroponically treating seedlings with four antitubulin compounds of diverse chemistry – colchicine, amiprophos-methyl (APM), a benzamide designated RH-4032, and a novel phenylcyclohexene colchicine mimic (2d), followed by

visual analysis of root tip morphology and cytological determination of the mitotic index, or fraction of cells at metaphase. An 18-hour treatment of 3-5 mM hydroxyurea gave optimum synchronization. Three of the antitubulin compounds, amiprophos-methyl, 2d, and RH-4032, outperformed colchicine, the standard agent for metaphase accumulation, and yielded average mitotic indices of 0.3. Peak mitotic indices exceeding 0.7 were observed for 2d and APM. Shoot bud topical application of APM, 2d, and RH-4032 produced short term changes ranging from sectored and misshapen leaves to meristem necrosis, followed by development of thicker and darker leaves. Cytological data and visual observations suggest these compounds cause somatic doubling of the chromosome number in cotton, and that one or more of them will provide a favorable alternative to the traditional methods based on colchicine, which is highly toxic and mutagenic to humans.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Dr. David Stelly, for his unwavering patience and forthcoming advice. I would next like to thank the graduate students, Brian Gardunia, Kelly Biddle, and Nilesh Dighe, who were always willing to troubleshoot and give suggestions no matter how often I needed them. I would also like to thank Wayne Raska, Dr. Keerti Rathore, and Dr. Spencer Johnston for technical expertise and resources for various aspects of the project. Finally I would like to thank Dr. Peggy Thaxton for providing seed, and Dr. David Young and Dr. Akio Kato for collaboration that made parts of this project possible.

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CHAPTER I

INTRODUCTION

Cell growth and replication is essential for all life and occurs in somatic tissues of eukaryotes by way of a complex system of events known as the cell cycle. During the cell cycle, a cell first doubles its cytoplasmic and nucleic content, and then carefully undergoes highly regulated division into two identical daughter cells. Control over this biological process is of great interest to modern biology. In medicine, countless approaches have focused on methods to inhibit the rampant and otherwise uncontrollable replication of cancer cells. In many branches of biology, including cell biology, pharmacology, agricultural chemistry, molecular biology, and pathology, there is also a need for control over the cell cycle, especially when a specific stage of the cycle is sought from among large populations of cells. Such control can allow researchers to enrich a large population for the desired stage. Because most somatic cell populations divide asynchronously and contain only a fraction of cells in a particular phase, enrichment for one stage requires that synchrony be induced artificially. In cytogenetics, prometaphase and metaphase are highly valued for karyotypic and fluorescent *in situ* hybridization applications that depend on slightly extended to maximally condensed chromosomes.

Cotton (*Gossypium hirsutum*) is an economically important fiber and oilseed crop.

This thesis follows the style and format of *Cell*.

Because of its value, genetic improvements are sought world over by conventional and biochemical means. Conventional improvements are addressed by a combination of breeding methods including interspecific germplasm introgression. Efforts to characterize the cotton genome are expanding domestically and internationally (International Cotton Genome Initiative, icgi.tamu.edu), and may eventually grow to include complete genome sequencing. Chromosome manipulation and cytogenetic analysis are essential to germplasm introgression and genomics. These and other tasks will be greatly facilitated in cotton by procedures that enable reliable production of high quality mitotic chromosome preparation for cytological analysis, and/or efficient chromosome doubling for ploidy manipulation in breeding. However, development of these capabilities in cotton has been impeded by the lack of suitable cell synchronization procedures. Thus, the primary goal of this project will be to develop a highly effective synchronization procedure for metaphase accumulation.

The second major goal of this research is to develop improved procedures for chromosome doubling in cotton. A large global range throughout its evolution and a history of widespread cultivation have given rise to both diploid Old World ($2n = 26$) and allotetraploid New World varieties ($2n = 52$). Differences in both ploidy and meiotic chromosomal affinity affect species combinations, such that most interspecific hybrid combinations are partially or highly infertile (Fryxell, 1979). In a number of combinations, chromosome doubling can be used to facilitate germplasm introgression. This is especially important for the most economically important species, *G. hirsutum* and *G. barbadense,* because their level of genetic diversity is extremely low and hinders genetic progress from breeding. Germplasm introgression increases heterozygosity and

allows beneficial traits, such as those conferring pest resistance and improved fiber quality, to be introduced into the cultivated species.

Artificial polyploidy by chromosome doubling can often be used to overcome sterility barriers arising from pairing difficulties arise due to non-homology between the parental species' homologous chromosomes. Chromosome doubling restores fertility to such interspecific hybrids by providing a pairing complement for each chromosome. Recent such accomplishments include wheat (Wojciechowska et al., 2002), *Arabidopsis* (Comai et al., 2000), and cucumber (Chen et al., 2003).

Both cell cycle synchronization and chromosome doubling typically involve the application of a mitotic spindle inhibitor, the most commonly used of which is colchicine. Multiple drawbacks to colchicine use exist, however. First, colchicine has proven to be notoriously inconsistent for plant cell cycle synchronization (Bordes et al., 1997) and chromosome doubling (Kato, 2002), and has been altogether unsuccessful for some species. For doubling purposes, death of the primary shoot generally results upon treatment of shoot meristems, and only a low frequency of doubled secondary shoots can be recovered. Secondly, colchicine is known to be much more potent toward mammalian cells than toward plant cells, making it a strong mammalian toxin that must be used with extreme caution. Moreover, this requires that it be used in relatively high concentrations for plants, thereby increasing potential for hazardous human contact. Alternatives to colchicine have attempted to avoid these drawbacks. Many antimicrotubule herbicides, such as oryzalin, trifluralin, amiprophos-methyl, and pronamide, have been identified as potentially valuable doubling agents. Of these, amiprophos-methyl (APM) appears to be the most effective agent for chromosome doubling in plants (Hansen et al., 1998).

Moreover, its apparent specificity for plant tubulin (Murthy et al. 1994) potentially reduces its toxicity to humans. Two novel chemicals, 3,5-dichloro-N-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-benzamide, designated RH-4032 (Young et al., 2000), and a phenylcyclohexene colchicine mimic designated 2d (Young et al., 2001), have also been developed with significantly increased specificity for plant tubulin over mammalian tubulin, although their capacity for cell cycle synchronization and chromosome doubling have not been analyzed. Another method of chromosome doubling is the use of nitrous oxide, a non-toxic gas that, in wheat (Hansen et al., 1988) and maize (Kato, 2002), has proven less laborious and as effective as colchicine, and is thus a potential candidate for cotton cell cycle synchronization and chromosome doubling.

Despite the above variety of doubling agents and procedures that are available, most researchers have found *in vivo* chromosome doubling to be a highly inconsistent and laborious process. Very little research on chromosome doubling in the cotton genus has been reported. It seems likely that much of the inefficiency is due to cytotoxic effects of chromosome doubling agents. In practice, the optimal duration of exposure to these chemicals is typically a balance between doubling and lethality, such that exposure levels often approach LD50. Since cells under treatment can only double during mitosis, which represents a relatively small fraction of the cell cycle, one way to increase doubling efficiency in the treated tissue would be to maximize the fraction of mitotic cells by imposing artificial synchronization. Surprisingly, no reports have analyzed doubling efficiency in populations of synchronously dividing cells.

Existing *in vivo* synchronization procedures are limited to root meristems, presumably because this is the easiest tissue to work with experimentally and hence the

obvious choice for cytogenetics applications. However, chromosome doubling with the goal of germplasm introgression will be most useful in shoot meristems, where doubled sectors can be propagated or used for seed production. In practicality, it seems likely that various chromosome doubling efforts will culminate on treatments aimed at *in vitro* cultures of intact meristems, so that the chemicals can be introduced and removed relatively quickly in a highly controlled environment. However, preliminary research need not be confined to *in vitro* cultures, and experiments may be conducted on shoot apical meristems, provided such a synchronization protocol can be developed. Synchronized meristems will theoretically produce an increased percentage of doubled progenitor cells upon chromosome doubling treatment.

CHAPTER II

CELL CYCLE SYNCHRONIZATION

A variety of methods have been developed to induce cell cycle synchrony in plant systems. Nutrient starvation followed by transfer to fresh medium has been used with some success in suspension-cultured cells (Arumuganathan et al., 1991). But synchrony is critically dependent on the state of the cells, so most methods employ DNA synthesis inhibitors that have nominal affects on cell viability. Treatment periods are generally longer than the length of the cell cycle, and result in accumulation of cycling cells that have been arrested in late G1 to early S phase (Figure 2.1). Thorough removal of the inhibitor allows the arrested cells to resume cycling in a synchronous manner.

One of the cheapest and most commonly used inhibitors is hydroxyurea, which targets the nucleotide biosynthesis pathway and DNA synthesis by reversibly inhibiting ribonucleotide reductase, and thus the production of dNDPs from rNDPs. Other inhibitors of DNA synthesis that work by a variety of mechanisms include aphidicolin, 5-aminouracil, and mimosine. Problems that may have contributed to their relatively underused status as synchronizing agents include variable efficacy of aphidicolin from different suppliers (Samuels et al., 1998), and significant induction of chromosome aberrations by 5-aminouracil (Navarrete et al., 1983). Mimosine appears to be the most infrequently used candidate for synchronization, despite its superior performance over hydroxyurea and aphidicolin in carrot cell suspension culture (Ghosh et al., 1999).

Following synchronization, cells can be accumulated in metaphase by a treatment that is antagonistic to chromosome movement. Disruption of the spindle is a common

approach, as it eliminates tension on centromeres and thus invokes a tension-reporting metaphase/anaphase cell cycle checkpoint. Most often, disruption of the mitotic spindle apparatus typically involves treatment with an antitubulin chemical and/or cold temperature. The length of the treatment generally correlates with the percentage of cells in arrested metaphase, although longer treatments are associated with chromosome decondensation and chromosomes splitting into chromatids (Dolezel et al., 1992). Moreover, cytotoxicity of the chemicals make extended treatment undesirable.

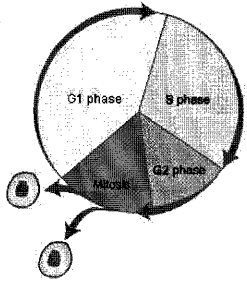

Figure 2.1. The cell cycle. (http://712designs.com/animations.htm)

Dolezel *et al.* (1999) reported a maximum mitotic index in *Vicia faba* between 50 and 70% using optimized hydroxyurea synchronization and APM metaphase accumulation. However, because more than 90% of cells in *V. faba* root meristems are cycling (Dolezel et al., 1992), and the treatment exceeded the length of the cell cycle, the efficiency of this procedure is at best 50 to 80%. This indicates that a large percentage of cycling cells are either irresponsive to the treatment, or are being prevented from

advancing to metaphase within the time period analyzed, or both. Presumably the latter explanation would be due to cytotoxic effects of the compounds. Variability in response can exist even in populations of cells that otherwise seem to be homogenous, which can effect synchronization efficiency. Samuels et al. (1998) reported that neighboring clumps of tobacco BY-2 cells in culture responded differently to synchronization, while cells within each clump tended to have similar responses.

A goal of the cell cycle synchronization part of this project was to ascertain if APM and other antimicrotubule compounds do indeed affect the cell cycle prior to metaphase, or to otherwise determine the cause of synchronization inefficiency. A bromodeoxyuridine (BrdU) based proliferation assay was incorporated within the synchronization procedure, and proliferation and synchronization were analyzed simultaneously on a cell-by-cell basis. BrdU is a thymidine analog that is incorporated into new DNA during DNA synthesis (S) phase of the cell cycle. Incorporated BrdU alters fluorescence emission from dyes that bind to nucleic acids, which allows detection of BrdU-labeled cells (Frey, 1994). Quenching of fluorescence by BrdU was used to distinguish cells that had undergone DNA synthesis during the recovery period, and thus were susceptible to APM-induced metaphase accumulation, from those that did not undergo synthesis and were unable to reach metaphase. Presence of interphase (G1, S, and G2) cycling cells (as denoted by their BrdU-label) indicated that either inefficiency of the metaphase block allowed advancement through metaphase, or toxicity halted the cell cycle prior to metaphase.

The proliferation assay is still under development, and further application should ultimately allow the elimination of unlabeled cells from further consideration, because,

theoretically, they were non-viable before APM-treatment began. Simultaneous analysis of proliferation and synchronization will be used to improve information about synchronization efficiency, since noncycling cells can be excluded from the estimates of mitotic index. An extremely important benefit may be that the proliferation assay could reduce error resulting from nonuniformity of seedlings and regions within individual root tips. The meristematic region of the root tip is small, and cycling rates could vary significantly between seedlings during the first few days immediately following germination.

Experimental Procedures

Germination

All cell cycle experiments were performed on *G. hirsutum* variety 96WD22 (courtesy of Peggy Thaxton, TAMU). Seeds were germinated in "ragdolls" by evenly spreading them on moist germination paper, each paper having been folded in half lengthwise. The germination papers with seeds were then loosely rolled and secured with a rubber-band two centimeters from the top, and soaked for at least ten minutes in a Banrot™ antifungal solution to prevent seed rot and to allow the seeds to imbibe water. The ragdolls were then allowed to stand, vertically with secured end up, in one centimeter of Banrot™ solution in the dark at 30° C for two days.

Synchronization

To establish the synchronizing effects of hydroxyurea, actively growing seedlings were selected and suspended with a pipette-tip holder (Figure 2.2) in 1X Hoagland's

solution containing various concentrations of hydroxyurea (Sigma). The seedlings were then placed back in the dark at 30° C with rapid bubbling for 18 hours. A fish tank aerator and plastic tubing were used for aeration, and were periodically cleaned with a dilute bleach solution. At the end of the 18-hour treatment, seedlings were rinsed vigorously with 30° C water prior to placement in fresh 1X Hoagland's solution. In experiments that included proliferation analyses, BrdU (Sigma) at 20-200 μM was included in the recovery solution. Primary root tips were harvested at various time points afterwards and fixed overnight in 3:1 (three parts ethanol, one part acetic acid).

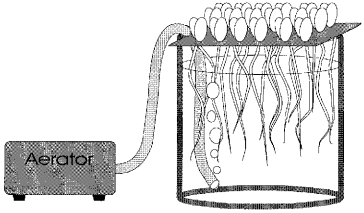

Figure 2.2. Treatment apparatus for synchronization.

Metaphase Accumulation

To analyze the effects of antimicrotubule chemicals, seedlings were treated as above, but aliquots of the respective chemicals in DMSO were added to the recovery solution two hours after removal from hydroxyurea. APM was obtained courtesy of Bayer Agriculture Division, Kansas City, MO. RH-4032 and 2d were obtained courtesy of David Young of Dow Agrosciences, Indianapolis, IN. To facilitate chemical penetration and improve consistency of results, DMSO was added to final concentrations

of between 0.05 and 0.1%. Root tips were then harvested and fixed as above at various time points.

Slide Preparation, Staining, and Microscopy

Chromosome preparations were made according to the methods of Kim et al. (2002), except for the pretreatments and slight modifications. Root tips were removed from fixative and placed in two changes of water for a total of at least 45 minutes. The one- to two-millimeter section behind the root cap was then excised with a razor blade and digested in a cell wall-hydrolytic enzyme solution (5% cellulose and 2.5% pectolyase in 0.1 M citrate buffer) at 37° C for approximately one hour, after which the root tips were suspended in water to remove the enzyme solution, and pelleted by centrifugation. The pelleted tips were then macerated in a small amount of 3:1 (volume adjusted to give the desired cell concentration), and a drop of the cell suspension was allowed to dry on a clean slide. Dried slides were then stained with azure B and analyzed by light microscopy using a 40X oil objective.

Root tips containing incorporated BrdU were instead stained for one hour with a solution of 15 μg/ml propidium iodide and 20 μg/ml Hoescht 33258 (Sigma) dissolved in PBS buffer, followed by a destaining period of at least one day to reduce excess cytoplasmic staining. Slides were analyzed with an Olympus AX-70 epifluorescence microscope using a short-bandwidth DAPI filter, for Hoescht fluorescence, and a Cy3 filter for propidium iodide fluorescence. Images from each filter were captured and combined using MacProbe v.4.2.3 digital imaging system (Applied Imaging Corp., Santa

Clara, CA). Because BrdU-labeled DNA is light labile, harvesting and handling of root tips was confined to the dark whenever possible.

Results and Discussion

Synchronization with Hydroxyurea

Each slide's mitotic index was calculated as the fraction of dividing cells (prometaphase, metaphase, and anaphase) in a total cell count of at least 300. Data were analyzed with SPSS 11.0.1. Note that bars for all graphs show mean mitotic indices, and error bars show the mean +/- 1.0 SE. Analysis of hydroxyurea concentration effect on mitotic index is shown in Figure 2.3 (A) and (B). Figure (A) represents combined data from three experiments using 3-4 mM hydroxyurea (HU), with samples taken from 3 to 5 hours. As can be seen, the peak of mitotic activity occurs around 4.5 hours following removal from hydroxyurea. Figure (B) represents combined data from three experiments, where bars are mean mitotic indices between 3.5 and 5 hours (samples taken at 30 minute intervals). These results demonstrate that hydroxyurea alone can be used between 3 and 5 mM to increase the mitotic index at least three fold over untreated root tips, with resulting slide preparations similar to that in Figure 2.4, which shows a high percentage of metaphase and anaphase cells at five hours after removal from 3.5 mM HU.

While a three-fold increase in mitotic activity is significant, it is not consistent with hydroxyurea's potent synchronization capacity as suggested by Dolezel et al., (1999), who used it alone in *Vicia faba* root tips to attain mitotic indices exceeding 50%. While species specific differences were noted, optimum HU concentrations never exceeded 2.5 mM, and it was suggested that higher concentrations lead to delayed

recovery from the block and poor synchrony. The relatively high HU concentrations required for cotton synchrony indicate that cotton is only weakly susceptible to HU-induced synchronization, and requires correspondingly high concentrations that also lead to unfavorable recovery. This could reflect the tetraploid nature of *G. hirsutum* in that genes coding for several variants of hydroxyurea's target enzyme, ribonucleotide reductase, could be expressed simultaneously within the genome. These results warrant analysis of other synchronizing agents, such as aphidicolin and mimosine, and combinations of synchronizing agents, which may be more effective in cotton.

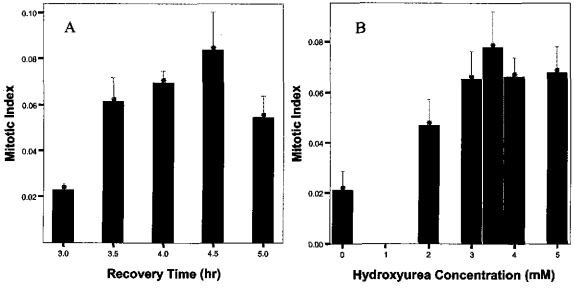

Figure 2.3. Analysis of length of recovery (A) and hydroxyurea concentration (B) for peak synchrony.

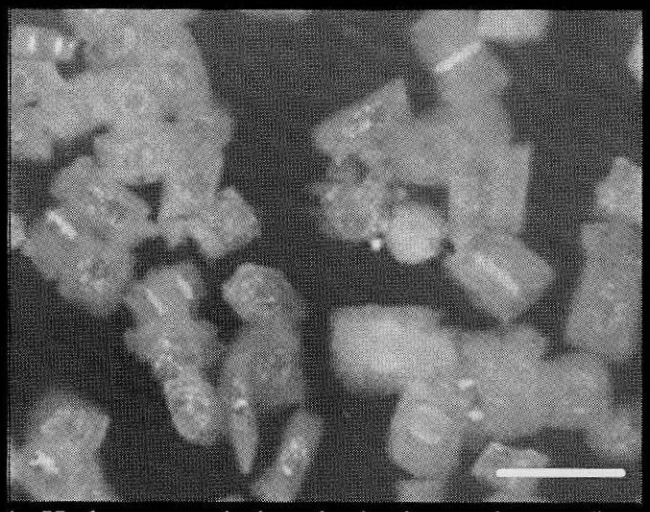

Figure 2.4. Hydroxyurea-induced mitotic synchrony. (bar = 50 μm)

Metaphase Accumulation with APM

Metaphase accumulation of cells enhanced the level of synchrony achievable by hydroxyurea treatment. Antitubulin chemicals disrupt polymerization of the mitotic spindle, causing failure of the chromosomes to align at the metaphase plate, and a delay at the metaphase/anaphase checkpoint of the cell cycle. APM was used to demonstrate the additional synchrony gained by temporary metaphase accumulation of synchronized cells (Figure 2.5). Cells depicted by graphs (A) and (B) were synchronized with 3.5 mM HU, while cells depicted by (C) were maintained in HU-free medium. At two hours after removal from HU, 10 μM APM was added to (B) and (C). Bars for each graph represent mean mitotic indices of three trials. Not all time points were collected for each experiment. The left- and right-hand graphs show the synchronization inducible by hydroxyurea alone and APM alone, respectively, while the middle graph shows the results of trials with both HU synchronization and APM metaphase accumulation. Peak mitotic activity occurred between 4 and 5 hours after removal from HU, corresponding to 2 to 3 hours of exposure to APM. Included in the 9-hour mitotic indices of both APM-

treated groups was a substantial proportion of decondensing metaphases. Metaphase accumulation by APM results in an additional three-fold increase in synchrony over HU-treatment alone, and APM treatment by itself produced mitotic indices intermediate those of HU alone and the combined (HU/APM) treatment. Figure 2.6 shows accumulated metaphase cells resulting from the hydroxyurea/APM treatment. Note the abnormal metaphase appearance due to chromosome aggregation.

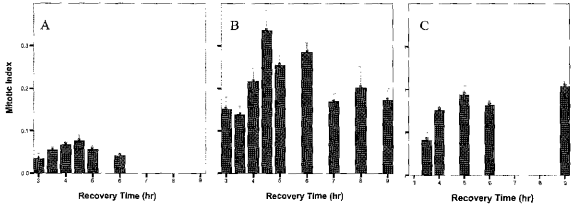

Figure 2.5. Enhanced synchrony by APM-induced metaphase accumulation. Synchrony from HU synchronization alone (A), APM-induced metaphase accumulation of HU-synchronized cells (B), and APM-induced metaphase accumulation of non-synchronized cells (C).

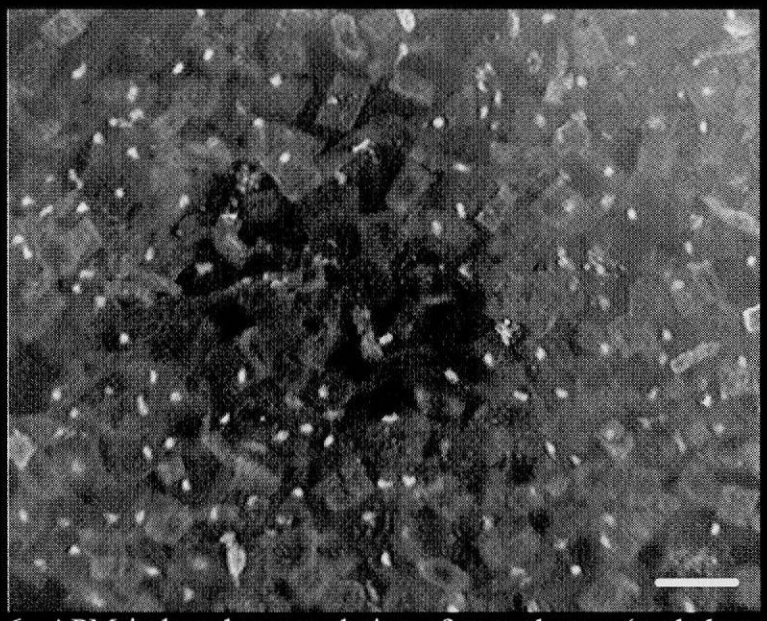

Figure 2.6. APM-induced accumulation of metaphases. (scale bar = 50 μm)

It is obvious from the above graphs that mitotic index does not increase linearly with duration of APM treatment, and that the metaphase block is only temporary. The peak mitotic index occurs at 4.5 hours of recovery in both (A) and (B), followed by a gradual decline. The similarity of these two graphs indicates a transient APM-induced block that lengthens metaphase only minutes more than non-inhibited metaphase. Likewise, graph (C), which would be expected to have a linear upward slope in the event of an indefinite APM block, instead levels out at a mitotic index of 0.2. The greater than four-fold increase that APM contributes to hydroxyurea-induced synchrony demonstrates the significance of the APM block, regardless of its short duration. In effect, APM integrates a small expanse of the HU-induced synchrony curve. Samples taken after 24-hour treatments (not shown) had reduced mitotic indices comparable to untreated control roots. Together, these data point to two possibilities, the most likely of which being that APM causes decondensation of metaphase chromosomes while concomitantly inhibiting further metaphase accumulation through toxic effects. The other explanation is that all

treated cells, regardless of their stage of the cell cycle, quickly develop a short-term resistance to, or deactivation of, APM. Such a resistance response to antitubulin chemicals has not been reported in the literature.

Proliferation Assay

One aspect of the synchronization procedure that has persisted despite uniform treatment and careful optimization of growing conditions is a high variability in results. While the average peak mitotic index inducible by APM was around 0.3, individual trials had values between 0.1 and 0.7. Moreover, subpopulations within single trials demonstrated a high degree of variability. Figure 2.7 shows two neighboring clumps of cells from a slide preparation of root tips that were in the same treatment of 3 mM HU/10 μM APM. Metaphases, visible as dense blotches, are much more frequent in the upper clump of cells. Due to either nonproliferation or phenotypic variability between root tips or neighboring regions within the meristematic region, such variability can be expected in an otherwise homogenous treatment sample.

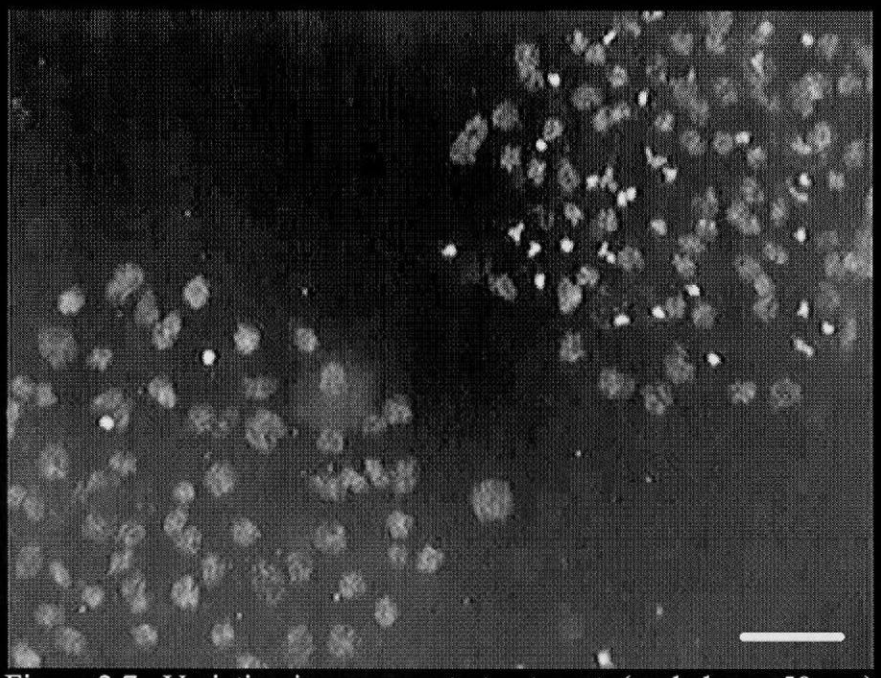

Figure 2.7. Variation in response to treatment. (scale bar = 50 μm)

Other questions that must be answered involve the declining mitotic indices at extended time points. Presumably, the cells cannot advance through metaphase without the mitotic spindle, but undoubtedly the non-metaphase population after the peak mitotic index is partially composed of previously metaphase cells. A temporary block would allow polymerization of the spindle and thus alignment of the chromosomes, corresponding to the reappearance of normal metaphases. The existence of normal metaphases declines sharply from 3-5 hours, and then, through the duration of the treatment, does not reappear. Thus, it appears that the chromosomes have decondensed without division, resulting in either chromosome doubling or an indefinite delay in progression through anaphase. What is the state of these post-metaphase cells?

A proliferation assay was developed, both to achieve more consistent results, and to answer some of the questions about individual cells' susceptibility to the chemicals. BrdU incorporation was used to label cells that had undergone DNA synthesis –

progressed through S phase – during recovery from hydroxyurea. The presence of BrdU in DNA quenches fluorescence emissions by certain DNA-binding dyes. Hoescht 33258 was chosen as the BrdU-susceptible dye because of its availability and preferential use in the recent literature. Likewise, propidium iodide was selected as a non-BrdU dependent control. Differential staining by these dyes distinguished noncycling cells from resistant, cycling cells, by the level of Hoescht quenching. To test for BrdU toxicity that could alter cell cycling rates, concentrations from 5 to 200 μM were analyzed.

Figure 2.8 (A) and (B) shows Hoescht and propidium iodide staining, respectively, of cells from hydroxyurea/APM treated root tips with 100 μM BrdU recovery solution. Figure (C) shows the combined image with BrdU-incorporated cells appearing purple, and non-BrdU-incorporated cells appearing blue. Figure (D) is a control slide of root tips receiving a non-BrdU-containing recovery solution. Metaphase cells were used as a reference in each image by adjusting relative intensities of each hue to give a uniform purple. Thus far, there seem to be no toxic effects of BrdU within the concentration range tested, and concentrations above 20 μM result in noticeable Hoescht quenching. Further analysis of synchronization in conjunction with this assay will determine its reliability and potential for improving the quality of synchronization data.

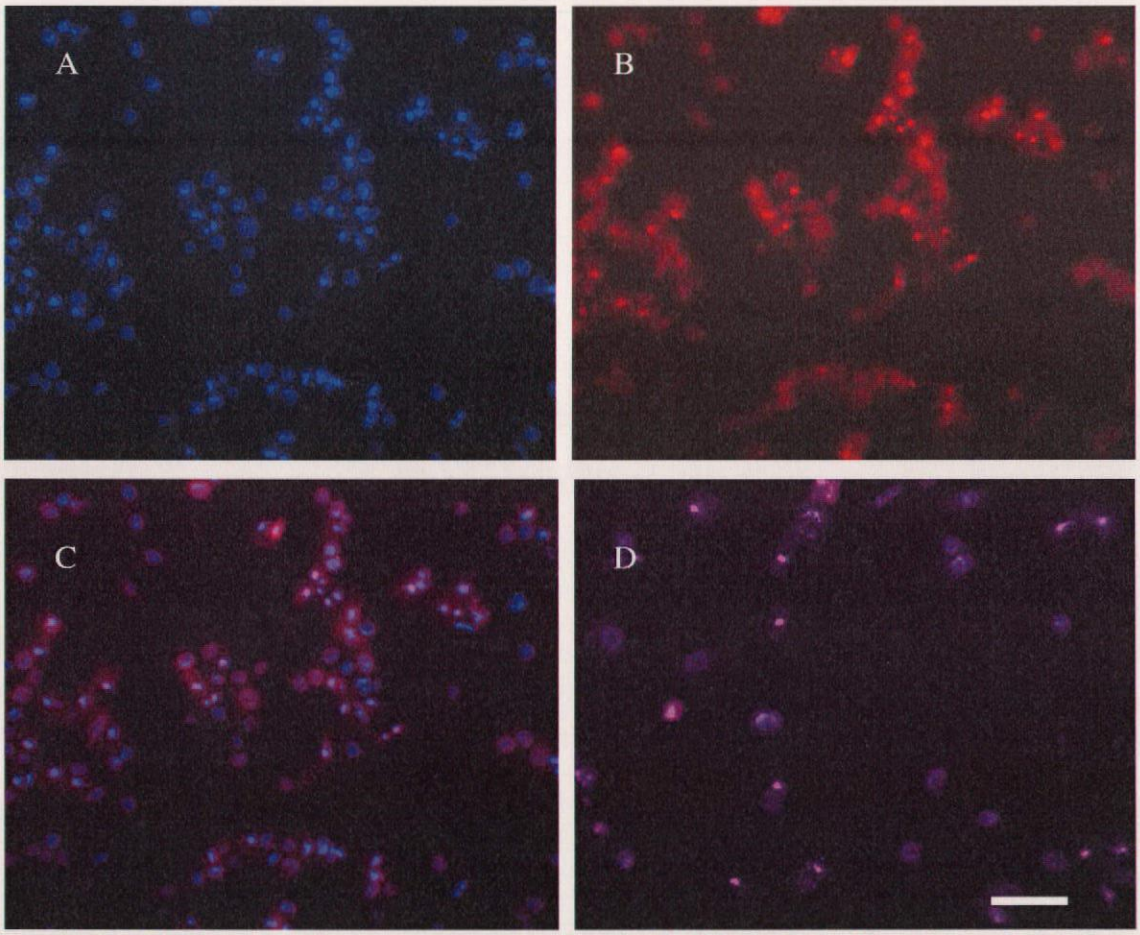

Figure 2.8. Differential fluorescence quenching in BrdU treated cells. (A) and (B) show fluorescence from Hoescht and propidium iodide, respectively. (C) is the combined image revealing Hoescht quenching. (D) is combined image of a control slide (no BrdU). (bar = 50 μm)

Comparison of Chemicals for Metaphase Accumulation

Comparisons were made with a variety of antitubulin chemicals in an effort to find the most effective agents and respective concentrations for metaphase accumulation. All chemicals demonstrated a time-dependent synchrony curve similar to that of APM in Figure 2.5. Treatments were made on HU-synchronized root tips, and mitotic indices at 4-6 hours of recovery were used for comparison of metaphase accumulation between chemicals (Figure 2.9).

As expected, colchicine had the poorest performance in the concentration range tested, with 2d, APM, and RH-4032 inducing higher mitotic indices at 10-fold lower concentrations. 2d was especially potent, yielding average mitotic indices above 0.3 at just 1 μM. Even at 0.5 μM, the lowest concentration graphed, 2d shows to have a significant effect. Note that the graphs do not include values for 0 μM treatments. Occasional instances of mitotic indices exceeding 0.7 were observed with both 2d and APM, although significant variability was inevitably due to the effectiveness of hydroxyurea synchronization between trials.

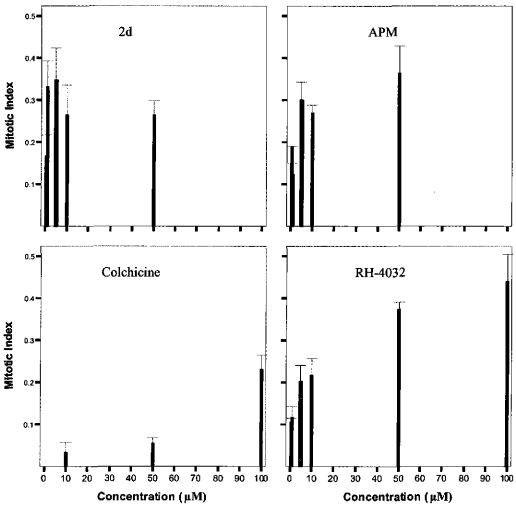

Figure 2.9. Analysis of antimicrotubule chemicals and concentrations.

Preliminary evaluations were made with nitrous oxide gas by Akio Kato, University of Missouri, Columbia, MO. Pressures of 600 kPa and 1000 kPa were used on young cotton seedlings. Many normal anaphase cells were observed in the 600 kPa treatment, indicating uninterrupted cycling, while the 1000 kPa treatment resulted in elevated mitotic indices. Further evaluations were not conducted due to significant toxicity of both treatments.

Attempts to Reduce Chromosome Clumping

Several reports on the use of microtubule disruptors have indicated the occurrence of chromosome clumping induced by these chemicals. For reasons that seem not to be addressed in the literature, treated metaphase chromosomes tend to aggregate tightly into clumps that make them virtually impossible to distinguish individually, and significantly hampers their separation by flow cytometry (Lysak et al., 1999). Lee et al., (1996) noted that chromosomes began to clump with trifluralin treatments over five hours, and recommended that treatments be kept as short as possible to minimize the problem. Lysak et al. (1999) and Dolezel et al. (1999) reported that overnight incubation of APM-treated roots in ice water improves chromosome spreading for various cereal species.

As seen in the aforementioned figures of accumulated metaphase cells (Figures 2.6-7), APM-treated cotton metaphase chromosomes demonstrated extreme susceptibility to the clumping effect of APM. Colchicine, RH-4032, and 2d also exhibited this unwanted side effect. If insurmountable, chromosome clumping severely challenges the usefulness of synchronization protocols developed for this species. In order to make

available the benefits of this cotton cell cycle synchronization procedure, existing methods of spreading chromosomes were evaluated.

Because treatment duration is a known factor, addition of APM was postponed for up to two additional hours during the recovery period, so that root tips could be harvested during the time of peak metaphase activity after as little as one hour of exposure to the accumulating agent. Other than significantly reducing mitotic indices, this approach had no effect on chromosome spreads.

Overnight incubation in ice-water was also analyzed because of its known chromosome spreading effect in cereal species. However, no effect on cotton chromosome spreading was observed from this treatment. Other tactics targeted slide preparation more directly, such as extended enzymatic digestion and acid hydrolysis to ensure thorough removal of the cell wall. These were followed by sequential suspension of the protoplasts in an elevated osmoticum solution (up to 20% high-molecular weight polyethylene glycol) and a reduced osmoticum solution (distilled water). Although this treatment resulted in larger cell volumes, it did not affect distances between chromosomes.

It seems that cotton represents a particularly troublesome species in this regard, and perhaps chromosome aggregation is a problem that cannot be avoided in procedures using antitubulin chemicals. The solution may indeed come from the use of chemicals with alternative mechanisms, such as nitrous oxide gas. Induced chromosome aggregation by antitubulin chemicals represents an interesting biological phenomenon that begs for further attention. Future studies are planned to assay the state of tubulin

polymerization, as the clumping could conceivably be a result of interacting truncated spindle fibers attached at the kinetochores.

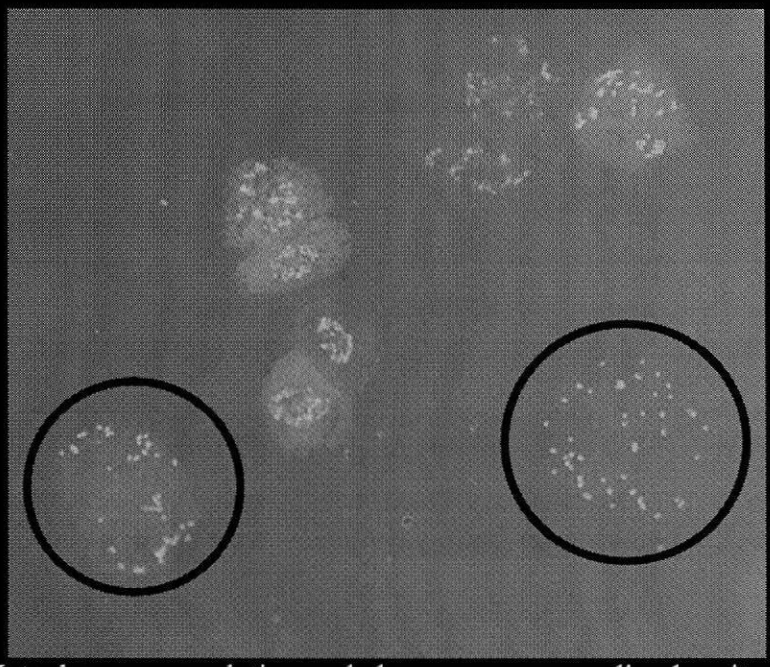

Figure 2.10. Metaphase accumulation and chromosome spreading by nitrous oxide. (courtesy of Akio Kato)

Though cotton did not respond well to nitrous oxide in preliminary trials, some potential was demonstrated for its future analysis as a cotton synchronizing agent. Nitrous oxide appeared to have a dispersing effect on cotton metaphase chromosomes (circled, Figure 2.10) in root tips that was consistent with previous observations made in other species (Kato, 1999). Consequently, the optimized use of nitrous oxide may circumvent the problem of chromosome clumping altogether.

CHAPTER III

CHROMOSOME DOUBLING

Several types of treatments can induce chromosome doubling in plants. Most of them involve extended treatment by chemicals that inhibit microtubule formation, leading to the metaphase/anaphase block and thus metaphase accumulation. The original and most popular antimicrotubule chemical for chromosome doubling in plants is colchicine. Physical methods such as heat-shock have also been attempted, but with little success. The goal is to produce chimeras with a doubled sector that can then be propagated separately. In the case of interspecific hybrids, an easily recognizable fertile tetraploid shoot will arise from an otherwise sterile diploid hybrid. More recently, this approach has been adapted for chromosome doubling of rice. *In vitro* culture treatments allowed induction of doubled haploids from ovules or anther culture, and the recovery of true-breeding homozygotes to be used in genetic analyses (Reiffers and Freire, 1990). Of course, the practicality of these experiments is greatly limited by *in vivo* applications, especially in species for which regeneration of plants is difficult, as in cotton.

The ultimate goal of this part of the research was to develop an *in vivo* chromosome doubling procedure for shoot meristems. But first, a chromosome doubling procedure in roots, which are a more defined experimental system, was used to test the hypothesis that synchronization can improve doubling efficiency.

Experimental Procedures

Germination, Treatment, and Analysis of Root Tips

Germination and treatment of cotton seed was conducted as in Chapter II. Both non-synchronized and hydroxyurea-synchronized roots were treated with 10 μM APM, 2d, RH-4032, and 500 μM colchicine beginning at 5 hours of recovery and lasting for either 8 or 24 hours. Following treatment, seedlings were rinsed thoroughly in distilled water and resuspended in 1X Hoagland's solution for 48 hours. New growth was then harvested and finely chopped in 1 mL Galbraith's buffer to release nuclei. Remaining plant material was filtered out and propidium iodide (2.5 mg/50 ml) was added. Samples were analyzed immediately by flow cytometry.

Preliminary Treatment and Analysis of Shoots

Infertile hybrid cotton plants (*G. raimondii* X *G. arboretum*) were propagated via air layering and maintained in a controlled-climate greenhouse. After five months of growth, they were pruned to approximately 0.3 m tall, and all apical meristems were removed. After one week of regrowth, rapidly growing shoot tips were treated with solutions of either APM, 2d, or RH-4032 at various concentrations determined in previous toxicity studies. Solutions contained 0.75% Latron AG-98 surfactant (Rohm and Haas) to enhance coverage and penetration. Applications were made once daily, in the afternoon, for one week. A drinking straw, with flow rate controlled by finger pressure on the top end, was dipped in solution and used to transfer treatment solutions to plants. Because 7 to 11 leaf initials are present above the first unfolded leaf (Kohel and Lewis, 1984), analyses of ploidy were made only after at least eight leaves had emerged

beyond each of the original, treated meristems. Visual screening was used to select potentially doubled plant sectors on the basis of leaf size, color, and morphology. Doubled tissue is expected to differ in appearance from normal tissue. Young leaves were harvested from the growing ends of each potentially doubled shoot, and flow cytometry was conducted as above.

Results and Discussion

Roots

Due to the availability of an optimized synchronization procedure, roots were used as the target tissue to test the hypothesis that synchronization will improve doubling efficiency. Roots following 24 hours of treatment were characterized by swelling just behind the meristematic region of the root tip, resulting in a club-shaped appearance of the roots. The 48-hour recovery period was generally long enough for the apical meristem to extend root growth beyond the swollen region (Figure 3.1), although in many cases 24 hours of treatment resulted in meristem death. No death was observed for 8-hour treatments.

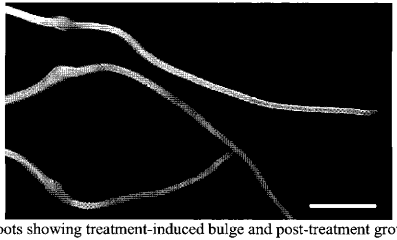

Figure 3.1. Roots showing treatment-induced bulge and post-treatment growth. (bar = 1 cm)

Presently, flow cytometry has not been conducted on non-synchronized treatment groups, so the effects of synchronization on doubling efficiency cannot yet be assessed. Flow cytometry on post-treatment growth of the synchronized group (Appendix A) suggests that both 8- and 24-hour treatments caused significant chromosome doubling, with nominal differences in doubling efficiency between the two treatment durations for APM and RH-4032. An interesting observation was the high proportion of doubling in the 10 μM 2d 24-hour treatment, which appeared to be caused by selective survival of doubled cells. The 500 μM colchicine treatment for 24 hours was severely toxic to roots and resulted in only one survivor. Pending repetition of these results in future trials, further analysis cannot be made at this time.

Preliminary results with nitrous oxide (Akio Kato, personal communication) indicate that obtaining doubled sectors will not be easy with this chemical due to severe toxicity of rapidly growing tissue at pressures necessary for doubling. Treatments were only conducted on non-synchronized meristems. It seems likely that toxicity can be reduced with shorter durations of nitrous oxide gas exposure, so potential exists for analysis of nitrous oxide treatment on synchronized tissue that will allow for shortened treatment durations.

Shoots

Pending thorough analysis of synchronization effects on doubling, and development of a synchronization method for shoot meristems, treatments were made directly to non-synchronized shoots to get a preliminary idea of each compound's doubling capacity. Shoots were much more resistant to the toxic effects of the chemicals,

even with the addition of surfactant, so concentrations of up to 1 mM APM and 100 μM 2d were used without significant lethality. Colchicine and RH-4032 were not analyzed. Flow cytometry confirmed that most shoots with altered coloration and morphology were indeed ploidy chimeras containing a significant percentage of doubled tissue. Size did not correlate well with ploidy chimerism.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

This research established a successful cell cycle synchronization procedure for cotton root tips that can be used reliably to attain mitotic indices of at least 0.3. Hydroxyurea at concentrations between 3 and 5 mM was able to increase mitotic indices three-fold to yield a mitotic index of around 0.1. However, higher degrees of synchronization are desirable, and other agents such as mimosine, aphidicolin, and 5-aminouracil deserve exploration. The degree of synchronization depends intimately on the careful control of physical parameters during the procedure. Consistent temperature and the absence of light are essential. The roots should be rinsed thoroughly after removal from hydroxyurea, as any carryover of HU into the recovery solution can lead to poor recovery. Additionally, the temperature of the rinse water should match that of the treatment solution.

Colchicine and a number of alternatives were explored for their capacity to accumulate synchronized cells at metaphase, with the conclusion that amiprophos-methyl and two recently developed chemicals, 2d and RH-4032, are highly effective for this procedure in cotton. These chemicals are more efficient and presumably safer to use than colchicine.

However, methods to prevent and/or disrupt chromosome clumping have thus far been ineffective. This raises serious concerns for the use of antitubulin chemicals for obtaining cytogenetics-quality metaphase cotton chromosomes. It is unknown why such a strong chromosome interaction develops in the presence of these chemicals. One

possibility is that aggregation is mediated by truncated microtubules arising from the kinetochores. Further research will be conducted into this phenomenon as well as alternative agents that do not induce clumping, with particular emphasis on nitrous oxide gas.

The proliferation assay being developed with this procedure will be useful in future analyses of synchronization. It will allow determination of individual cells' susceptibility to metaphase accumulation, thereby giving more accurate mitotic indices as well as useful information about the effects of individual chemicals on cell viability.

Extended treatments of both roots (APM, 2d, RH-4032, colchicine) and shoots (APM, 2d) resulted in ploidy chimeras for all chemicals tested. Preliminary results gave some indication that synchronization can be used to increase doubling efficiency, especially in lieu of the toxicity that was observed in most extended treatments with roots. Methods of shoot cell cycle synchronization are in development to allow doubling of synchronized shoot meristem cells. Approaches will initially focus on using a modified root-synchronization procedure, treating shoots either by inversion into the solution or by encapsulation within a treatment vesicle.

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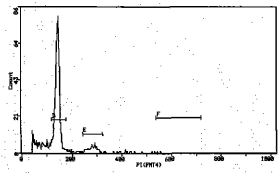
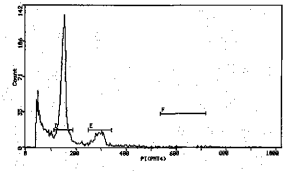
APPENDIX A

FLOW CYTOMETRY RESULTS OF TREATED SYNCHRONIZED ROOTS

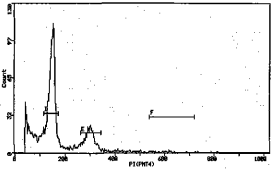
Control



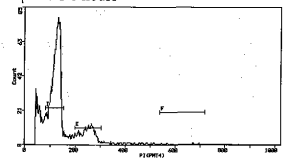
10 µM APM 8 hours



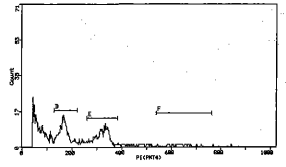
24 hours



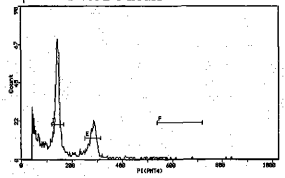
10 µM 2d 8 hours



24 hours



10 μM RH-4032 8 hours



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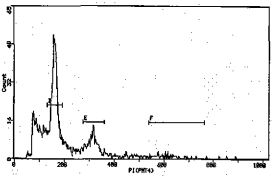
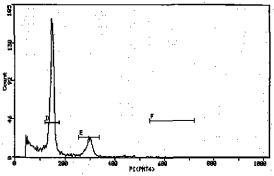
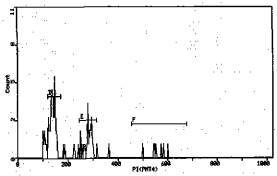


500 μM colchicine 8 hours



24 hours



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Presentations

"Improved Cell Cycle Synchronization and Chromosome Doubling Methods in Cotton." Student Research Week, Texas A&M University, College Station, TX. 3/30/2004

"Towards Improved Cell Cycle Manipulation and Chromosome Doubling Methods in *Gossypium*." (poster) Plant and Animal Genome XII, San Diego, CA. 1/14/2004

"Induction of Chromosome Doubling in an Interspecific *Gossypium* Hybrid" (poster) Beltwide Cotton Improvement Conference, San Antonio, TX. 1/8/2004

"A Comparison of Psl1 Candidate Gene Expression in Mouse Epidermis." Intern Research Symposium, UT MD Anderson Science Park, Smithville, TX. 8/15/2002