WIDE-CROSS WHOLE-GENOME RADIATION HYBRID (WWRH) MAPPING AND IDENTIFICATION OF COLD-RESPONSIVE GENES USING OLIGO-GENE MICROARRAY ANALYSIS IN COTTON

A Dissertation

by

WENXIANG GAO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Genetics

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December 2003

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ABSTRACT

Wide-cross Whole-genome Radiation Hybrid (WWRH) Mapping and Identification of Cold-responsive Genes Using Oligo-gene Microarray Analysis in Cotton.

(December 2003)

Wenxiang Gao, B.S.; M.S., Yangzhou University

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The first part of this research focused on wide-cross whole-genome radiation hybrid (WWRH) mapping of the cotton (*Gossypium*) genome. Radiation hybrid mapping has been used extensively to map the genomes of human and certain animal species, but not plant species. In lieu of *in vitro* hybrid cell line technologies for plants, we developed a novel approach for radiation hybrid mapping based on wide-cross *in vivo* hybridization. Flowers from one species of cotton, either *G. hirsutum* or *G. barbadense*, were γ-irradiated and then used to pollinate the other species. The resulting hybrid plants were assessed as a mapping tool. Two WWRH mapping panels were constructed from 5- and 8-krad γ-irradiation treatments. Both panels demonstrated that the WWRH mapping method can be used to map the cotton genome, and that this method complements traditional linkage mapping approaches.

The second part of this research focused on the identification of cold-responsive genes using spotted oligo-gene microarray analysis. Increased cold-tolerance in cotton would

promote early and uniform seedling establishment, expand the growing season, decrease susceptibility to fungal infections and certain diseases, and increase fiber yield and quality. BLAST searches of the cotton database using amino acid sequences of 93 drought/cold-related genes from *Arabidopsis* and several other plant species led to 806 cotton orthologous cDNAs and expressed sequence tags (ESTs). Eight hundred and six cotton 70-mer oligos were designed and included in an oligo-gene microarray containing 1,536 70-mer oligos, each representing a cDNA or EST from cotton, or one of 121 chloroplast genes or 66 mitochondrial genes from *Arabidopsis*. Thirty-eight cotton cDNAs and ESTs were identified as cold-responsive genes based on experimental treatment and oligo-gene microarray analysis. Expression was up-regulated for 36 genes and down-regulated for two genes by cold treatment. Results from microarray analysis were tested and confirmed by northern blot analysis for 16 genes. Our data suggest that *Arabidopsis* orthologous genes can be used to identify homologous cotton genes. The oligo-gene microarray is a valid approach to study transcriptional changes in cotton.

DEDICATION

I dedicate this dissertation to my wife, Qing Zhu, my daughter, Xing Gao, my parents-in-law, Qingchang Zhu and Junfang Liu, and my parents, Yuli Gao and Jing Chen, whose support, patience, and love have encouraged me throughout my graduate study.

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CHAPTER I

INTRODUCTION

Part I: The feasibility of wide-cross whole-genome radiation hybrid (WWRH) mapping of cotton

Cotton (*Gossypium* spp.) is the world's most important textile fiber crop. The cotton genus, *Gossypium*, is distributed throughout tropical and subtropical regions of the world. Of the 50 recognized species, most have 26 chromosomes (2n = 26) and are widely regarded as diploid, but five found naturally in the New World are 2n = 52 and regarded as tetraploid. Cytological analyses of chromosome size and meiotic affinity revealed that each 13-chromosome genome could be assigned to a genomic group (A, B, C, D, E, F, G, or K), and that the groups are distributed in a geographically related manner (BEASLEY 1940, 1942; PHILLIPS and STRICKLAND 1966; EDWARDS and MIRZA 1979; ENDRIZZI et al. 1984; STEWART 1995; PERCIVAL et al. 1999). The A-genome group occurs among African-Asian species and the D-genome group occurs among species in the New World. All of the 52-chromosome species are disomic (KIMBER, 1961), have an AD-genome composition, and are hybridized readily to form relatively

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This dissertation follows the style and format of Genetics.

fertile hybrid progenies (BEASLEY 1942; ENDRIZZI et al. 1985; PERCIVAL et al. 1999).

Genome maps offer extensive advantages for many sorts of genetic endeavors, such as integrative genomics, map-aided gene cloning, and marker-assisted breeding. Unfortunately, public maps of the cotton genome are relatively immature as compared with major crops such as rice (*Oryza sativa* L., 2n = 2x = 24) (GOFF et al. 2002; YU et al. 2002), corn (*Zea maize* L., 2n = 2x = 20) (http://www.maizemag.org), and wheat (*Triticum aestivum* L., 2n = 6x = 42) (http://wheat.pw.usda.gov/ggpages/maps.shtml#wheat). In fact, the number of linkage groups in available public linkage maps of tetraploid cotton still exceeds its gametic chromosome number (26), and numerous linkage groups have not been assigned to chromosomes (http://demeter.bio.bnl.gov/acecot.html; REINISCH, et al. 1994; SHAPPLEY et al. 1998; YU et al. 1998; ULLOA and MEREDITH 2000; ZHANG et al. 2002; MEI et al. 2003). Even today, cytogenetic stocks are the only means by which all chromosomes of cotton can be identified.

Among the factors that have hindered cotton genome map development are [1] a very high rate of recombination in cotton, [2] nonrandomness of crossover distribution, [3] reliance on genome-wide marker development strategies, [4] incomplete genome coverage with hypo-aneuploids suited to physical mapping, [5] under-utilization of molecular cytogenetic physical mapping, and [6] relatively low levels of *intra*-specific molecular marker polymorphism within the two cultivated tetraploids, *G. hirsutum* and *G. barbadense*. Because rates of *inter*-specific polymorphism are much higher,

interspecific hybridization has been used in *Gossypium* to facilitate development of larger numbers of polymorphic marker loci and thus denser maps (REINISCH et al. 1994; PATERSON and SMITH 1999; ZHANG et al. 2002; MEI et al. 2003). As might be expected, marker segregation distortion was observed in linkage mapping populations of interspecific hybridization (REINISCH et al. 1994; ZHANG et al. 2002; MEI et al. 2003). But marker segregation distortion was observed also in an intraspecific *G. hirsutum* mapping population (SHAPPLEY et al. 1998).

Alternative forms of genome mapping such as physical mapping are especially valuable in genome regions having very high or very low rates of recombination, because such regions are difficult to resolve by segregation analysis. In regions of high rates of recombination, a larger number of polymorphic markers are required to close gaps in linkage maps. High rates of recombination in *G. hirsutum* and other *Gossypium* species were first documented by cytological analyses of meiotic pairing in species, interspecific hybrids, and *G. hirsutum* chromosome aberration heterozygotes (SKOVSTED 1934; BEASLEY 1942). The numerical estimates of tetraploid cotton genome map length was 4600 cM or higher (STELLY 1993; REINISH et al. 1994). Given that the genome of *G. hirsutum* contains about 3161 Mbp (BENNETT and LEITCH 2003), one cM is equivalent to 0.69 Mbp on average. Even though cotton has a genome much larger and more complex than the model plant species *Arabidopsis thaliana*, the DNA content per cM is similar (MEINKE et al. 1998; PATERSON and SMITH 1999).

In genomic regions of low recombination frequency, physical mapping approaches are likely to be more efficient than recombination-based methods (e.g., linkage mapping) for robustly establishing linear order. With linkage maps, such regions can occur as segments of relatively high marker density and incomplete resolution. Such regions are seen in many higher density maps, including some linkage groups of cotton (REINISCH et al. 1994; ZHANG et al. 2002; MEI et al. 2003). They can arise from a lack of reciprocal recombination or non-recovery of recombinants. Most public cotton genome maps and mapping resources are relying on the use of interspecific crosses between *G. barbadense* and *G. hirsutum* to achieve high rates of polymorphism. These run an empirically predictable but presently nondefined risk of inversions, distortions, and other factors that can compromise the effectiveness of linkage analysis for genome mapping. The most effective approach will be one in which linkage maps are integrated with each other and with orthogonal physical mapping data to achieve complementarity.

In principle, physical mapping approaches could help resolve limitations in cotton genome mapping that have resulted from non-random crossover distribution. Although molecular data on crossover distribution in the cotton genome are limited, nonrandomness is clear. The two subgenomes of the tetraploid *Gossypium* species, A *vs.* D, are markedly different in DNA content (GEEVER et al. 1989) and chromosomal size (SKOVSTED 1934; BEASLEY 1942; KIMBER 1961), but their linkage lengths are similar (REINISCH et al. 1994). These differences translate grossly to differences in cM/Mbp at the chromosome level, because we know that the A- and D-subgenomes of AD

tetraploids are closely related to those of certain extant A- and D-genome 2n = 26 species, for which C-values differ almost 2-fold (STELLY and PRICE, personal communication). Moreover, the virtual absence of cotton metaphase I bivalent configurations with chiasmata near the centromeres clearly indicates that recombination in cotton is highly nonrandom at subchromosomal levels.

The difficulty of reducing the number of cotton linkage groups to the gametic chromosome number reflects a need for physical mapping. Where hypo-aneuploid cytogenetic stocks are lacking, most efforts to distinguish and identify high-recombination regions have relied on large-scale marker development strategies that are genome-wide, and not targeted to the specific segment. Even though the molecular cytogenetic methods for cotton were among the first established and most advanced of crop plants, including the use of bacterial artificial chromosome (BAC) clones for fluorescent in situ hybridization (FISH) in plants or animals (HANSON et al. 1995), molecular cytogenetic methods have been little used for chromosomal identification or coalescence of syntenic cotton linkage groups. Instead, the need for relatively few markers in certain high recombination regions of cotton continues to be largely addressed by additional conventional linkage mapping and large-scale marker development on a genome-wide scale. A more effective and more efficacious solution would be to include physical mapping as part of the overall strategy.

Improved resolution and integration of cotton genome maps are desirable. Several methods have been suggested to increase map resolution. One of them is radiation hybrid mapping (COX et al. 1990; WALTER et al. 1994). Goss and Harris (1975) first used radiation treatment to increase chromosome rearrangements between the human chromosome X and rodent chromosomes. After lethal X-ray irradiation of human cell lines, fragmented human X chromosome segments were rescued by rodent cells by means of cell fusions. In contrast to natural recombination, radiation-induced recombination frequencies and map resolution can be modulated by modifying radiation dosages. However, this technique for physical mapping was not widely employed until Cox et al. (1990) used radiation hybrids for the construction of a high-resolution map of human chromosome 21 and termed this technology as radiation hybrid (RH) mapping. Radiation hybrid mapping not only increased the recombination events, but it offered the advantages of very high rates of polymorphism between donor and recipient cell lines. Thus, it became possible to use markers that are monomorphic within the donor species for mapping (COX et al. 1990).

A major limitation of the RH mapping method of Cox et al. (1990), however, is that it maps only one chromosome at a time. Given large population sizes about 200-300 cell lines for relatively high resolution, it is difficult or impractical to generate a high-resolution map of organisms with large genomes (WALTER et al. 1994; RIERA-LIZARAZU et al. 2000). To overcome this limitation, Walter et al. (1994) first published an improved RH mapping method, termed whole-genome radiation hybrid (WGRH)

mapping. In WGRH mapping, whole-genome radiation hybrids are generated when the donor material is obtained from a diploid cell line of the donor species, rather than from a hybrid somatic cell single-chromosome addition line (COX et al. 1990; WALTER et al. 1994). As a result, the WGRH mapping method allows all chromosomes to be mapped using a single radiation hybrid panel (WALTER et al. 1994). The WGRH mapping approach has been assimilated into genome mapping efforts for humans and certain animal species (MCCARTHY 1996; SCHMITT et al. 1996; MCCARTHY et al. 1997; WOMACK et al. 1997; KWOK et al. 1998, 1999; PRIAT et al. 1998; YERLE et al. 1998; GEISLER et al. 1999; HAWKEN et al. 1999; MURPHY et al. 1999; VIGNAUX et al. 1999; WATANABE et al. 1999; KIGUWA et al. 2000; REXROAD et al. 2000; OLIVIER et al. 2001).

The rapid and extensive use of physical mapping attests to its value and the fact that there is a general need for mapping methods complementary to segregation analysis. Genome-wide and regional studies indicate distributions of recombination or crossing-over are generally nonrandom (reviewed in PUCHTA and HOHN 1996; COPENHAVER, et al. 1998; GERTON et al. 2000; reviewed in PETES 2001; YU et al. 2001; ISLAM-FARIDI et al. 2002). Chiasmata distributions, recombination nodule distributions, recombination-specific protein distributions, and integrated maps indicate that meiotic crossover distributions are extremely to moderately nonrandom in many if not all higher eukaryotes.

In plants, radiation treatments have been used mainly for gene mutation and gene transfer (DRISCOLL et al. 1963; KOHEL 1973; ISLAM et al. 1981; RILEY et al. 1984). Radiation-induced deletion mapping has occasionally been used in plants, e.g., in cotton, to induce deficiencies of whole chromosomes, arms, specific qualitative trait loci or linkage groups (KOHEL 1973; ENDRIZZI et al. 1984; STELLY and RASKA, personal communication). More recently, RH mapping of an oat-maize addition line was used to map maize chromosome 9 (RIERA-LIZARAZU et al. 2000). In the oat-maize system, the scope of mapping was relegated to single chromosomes, similar to the RH method of Cox et al. (1990). The difficulty of finding appropriate recipient cell lines to rescue irradiation-fragmented plant chromosomes seems to be a major obstacle in the application of *in vitro*-based RH or WGRH methods in mapping plant species.

Radiation-based methods are not the only approach and may not be the most effective approach for physical mapping, but they are relatively simple, modulated, scalable, and reasonably accessible. Other approaches that might be used include chemical mutagenesis (MCCALLUM et al. 2000; COLBERT et al. 2001) or the use of mutants (MYLES et al. 1989). It is quite feasible that these methods could be effectively used similar to radiation, alone or in combination with each other. However, the simplicity and widespread accessibility of gamma-irradiation, and the facility of modulating treatment dosages are important advantages.

To circumvent the limitations of radiation hybrid mapping in plants and expedite integrative mapping in cotton, I attempted to rescue the segmented genome of one species by hybridization with a different but reasonably closely related species, i.e., to use wide-cross *in vivo* sexual hybridization rather than *in vitro* somatic hybridization to introduce the segmented genome. To minimize mosaicism within panel components, I opted to introduce the fragmented genome through pollen. To denote this modified WGRH mapping approach, we have dubbed it as "*wide-cross whole-genome radiation hybrid (WWRH) mapping*". I will examine the feasibility of WWRH mapping of the cotton genome.

Part II: Stress-induced gene expression in cotton (*Gossypium hirsutum* L.) using 70-mer oligo-gene microarray analysis

Plants grow in diverse environmental conditions under various biotic and abiotic stresses throughout their life cycles. The abiotic environmental stresses that significantly affect plant and crop production worldwide include drought, cold and salt. Plants exhibit morphological, physiological, cellular and metabolic changes in response to environmental stresses. Plant responses to stress depend on duration and severity, genotypes, tissue types, and developmental stages. Some plant species have developed proper mechanisms to tolerate or to avoid environmental stresses. For example, some cotton species can tolerate dry seasons by developing deep-penetrating and extensive root systems, okra-shape leaves, and/or dropping leaves. *G. aridum* species drop their

foliage prior to flowering and fruit development. At the molecular levels, changes in gene expression are observed in plants under various environmental stresses (SKIVER and MUNDY 1990; BRAY 1991, 1994). The majority of responses to the environmental changes are predicted to require alterations in gene expression (WEISER 1970; BRAY 1994). Changes in gene expression in response to stresses have been studied in many crops such as maize (ZINSELMEIER et al. 2002), wheat (SIVAMANI et al. 2000), rice (SALEKDEH et al. 2002), tomato (KAHN et al. 1993), tobacco (FERRARIO-MERY et al. 1998) and cotton (ROARK et al. 1973; GALAU et al. 1992; PENNA et al. 1998). Different crops may have different responses to environmental stress. The development and yield of many crops can be repressed severely even by moderate water stress (HEATHERLY et al. 1977).

Environmental stresses are mainly studied by the differential screening methods using mRNA, protein or cDNA from stress-treated and non-treated plant materials. There have been studies on: (1) alterations in protein synthesis; (2) changes in mRNA transcript profiles shown by *in vitro* translations; and (3) characterization of the induced genes using comparative or subtractive cDNA library technologies. The cDNA library technique has been widely used because when a gene is identified, DNA and predicted amino acid sequence of the gene product can be obtained. The expression patterns of the cDNA associated with conditions required for accumulation of the mRNA and *in situ* localization within the organ can be studied. Thus, the most frequently used strategy to isolate stress-induced genes is differential screening of cDNA libraries. A given stress

condition may alter the expression for a set of genes, whereas related genes may be induced by different stresses. For example, some drought-induced genes are expressed (1) under cold and salt conditions, (2) after ABA treatment, and (3) during late stages of embryogenesis (BAKER et al. 1988; KAHN et al. 1993; YAMAGUCHI-SHINOZAKI and SHINOZAKI 1994; MANTYLA et al. 1995; XU et al. 1996; BRAY 1997; SHINOZAKI and YAMAGUCHI-SHINOZAKI 2000; TAJI et al. 2002). Stress-related genes often possess conserved sequence compositions and/or domains. Conserved stress-responsive pathways exist among plant species (JAGLO et al. 2001; HORVATH et al. 2003). Identified stress-induced genes can be grouped into two categories: (1) directly protecting plants against environmental stress and (2) regulation and signal transduction in response to environmental stresses (BRAY 1997; SHINOZAKI and YAMAGUCHI-SHINOZAKI 1997; THOMASHOW 1999; HASEGAWA et al. 2000).

Increased stress-tolerance levels can be genetically engineered in plant species. Transforming stress-tolerance genes and reprogramming the expression patterns of endogenous genes can improve stress tolerance. Treating plants with non-freezing low-temperature increases plant freezing tolerance in many plant species by inducing groups of genes to express under relatively low temperature (THOMASHOW 1999). Induced expression of the transgene encoding the transcription factor Dehydration-Responsive Element Binding-1A results in increased tolerance to drought, cold, and salt conditions (KASUGA et al. 1999). Overexpression of the transcription factor C-Box Binding Factor-1 leads to an increased cold stress tolerance (JAGLO-OTTOSEN et al. 1998).

Many tropical and subtropical plant species are sensitive to cold while plants originating from temperate zone are more tolerant to cold. Plant tolerance to cold and freezing is a complex trait because many genes are often involved (THOMASHOW 1990). About 1,000 cold-responsive genes have been expected in *Arabidopsis* (FOWLER and THOMASHOW 2002). Understanding plant cold tolerance process requires a comprehensive evaluation of gene expression on a large number of cold-inducible genes. Although some genes have been identified using traditional library screening methods and forward genetic approaches (THOMASHOW 1999), many cold related genes have been identified using high output techniques such as DNA microarray technology (FOWLER and THOMASHOW 2002).

Microarray technology has been recently used to analyze transcriptome changes induced by various environmental stress conditions (SEKI et al. 2001; KREPS et al. 2002; OONO et al. 2003). An advantage of using microarray technology is its ability to study changes in gene expression at a genome-wide scale, which makes it an excellent approach to study complex traits. Microarray analysis has been widely used in functional genomic studies, especially for the analyses of differentially expressed genes. Spotted microarrays use oligonucleotides or cDNA amplicons that can be hybridized simultaneously to two fluorochrome-labeled probes derived from different treatments such as cold-treated and non-treated plants. Two-color microarray analysis greatly facilitates the kinds of comparative analysis that are needed to study differential gene expression. To study

differential gene expression patterns on a large scale, cDNA or cRNA probes generated from stress-treated and non-stressed (control) tissues can be labeled with different fluorochromes, and then hybridized to common microarrays. Differences in expression between two treatments are based on differences in microarray color patterns. Various types of microarrays have been used to study the model plant species *Arabidopsis*, the genome of which has largely been sequenced. Nearly all of the *Arabidopsis* genes have been annotated, and functions of many genes have been determined.

To study and characterize genes within non-model organisms is more difficult than in *Arabidopsis*. One of the contributing factors is that most plants have more complex genomes than *Arabidopsis*. *Arabidopsis* has just 10 chromosomes (2n = 2x = 10) and a genome size (1C) of about 172 Mb (BENNETT and LEITCH 2003). Most economically important crop species have much larger and more complex genomes. For example, ninety percent of the cultivated cotton is *G. hirsutum*, which is 2n = 4x = 52 and has a genome size (1C) of 3161 Mb (BENNETT and LEITCH 2003). Therefore, genomic studies of crop species are far behind those of *Arabidopsis*. With the development of large-scale gene-expression analysis techniques such as microarrays (SCHENA et al. 1995; LOCKHART et al. 1996), many genes have been characterized in model organisms (SEKI et al. 2001; KREPS et al. 2002; OONO et al. 2003). Microarray capabilities are well developed and improving quickly in *Arabidopsis*, but much less so in other species. Applying microarray technology and information from model organisms to non-model organisms needs more attention. *Arabidopsis* microarrays have been used directly to

study other plant species. Horvath et al. (2003) used *Arabidopsis* cDNA microarrays to study developmentally differentiated genes in distantly related species. Better results would generally be expected if microarrays were developed directly from target species. No cotton microarrays have been reported.

Cold stress is among the most important abiotic stresses in cotton production. Cotton is regarded as a cold-sensitive or cold-susceptible crop, especially at the stages of seed germination, seedling, and flower development. Exposure of cotton at early developmental stages to cold often renders it susceptible to many other environmental stresses such as fungal infection. Increasing cold tolerance may expand the cotton growing season, increase tolerance to seedling diseases and other stresses, and consequently increase fiber yield and quality. In contrast to hundreds of cold-responsive genes or ESTs identified from *Arabidopsis* (SEKI et al. 2001; KREPS et al. 2002; FOWLER and THOMASHOW 2002), only a few drought- and possibly cold-tolerance genes have been reported in cotton. All were identified by traditional genetic methods from drought-stress experiments. Genes D7, D11, D19, D29, D34, and D113 are thought to be induced by drought stress in vegetative tissues, and genes D71, D131 and D147b are induced by drought stress on embryos (BAKER et al. 1988). Little is known about the function of those genes. Identification of cotton genes related to cold-tolerance will facilitate future development of cold-tolerant cotton cultivars through breeding or transgenic approaches (JAGLO-OTTOSEN et al. 1998; KASUGA et al. 1999; THOMASHOW 1999).

Comparative genomics and DNA sequence analysis indicate that many genes and gene functions are conserved during evolution. Many genes with similar functions have conserved domains, even among different species. For example, it has been demonstrated that all cereal plants studied contain homologs of the low-temperature-regulated gene, *WCS120*, first isolated in wheat (SARHAN et al. 1997). Genes induced by cold stress are often induced by drought stress (SEKI et al. 2001; KREPS et al. 2002). In this study, I will identify cotton cold-responsive genes through the analyses of 70-mer oligo-gene microarray designed based on cotton ESTs and cDNA with homology to known drought- and/or cold-related orthologous genes from *Arabidopsis* and other plant species.

CHAPTER II

WIDE-CROSS WHOLE-GENOME RADIATION HYBRID (WWRH) MAPPING OF COTTON (*Gossypium hirsutum* L.)

Radiation hybrids have been used widely to map genomes of human and certain other animal species, but little used in plant species. We report a modified radiation hybrid genome mapping approach for cotton (*Gossypium hirsutum* L.). Radiation hybrid mapping of human and animal genomes has relied on *in vitro* radiation hybrid cell lines that comprise individual radiation hybrid panel targeted at specific chromosomes or whole genomes. In contrast, we created *in vivo* "wide-cross whole-genome radiation hybrid" (WWRH) panels in which we used the genome of *G. barbadense* (n = 26) to rescue radiation-segmented genomes of *G. hirsutum* (n = 26) introduced via γ-irradiated (1.5, 5, 15 or 30 krads) pollen. After preliminary observations on seed production, germination and genotyping with 33 SSR markers from four chromosomes and one linkage group, we selected the 5-krad treatment for more detailed examination. To characterize retention characteristics, we screened 101 5-krad WWRHs with eight microsatellite markers in cotton linkage group 9 (LG-9). Relatively high marker retention frequencies (87% - 94%) resulted from the 5-krad γ-ray treatment, i.e., approximately equivalent to the rate of *loss* in a low-dose animal radiation hybrid panel. Co-localized SSR markers, BNL0625 and BNL2805, in traditional linkage map, were ordered along LG-9 by WWRH mapping. The marker distance correlation coefficient

between traditional linkage map and the WWRH map for LG-9 was 0.49. A 5-krad γ-ray WWRH mapping panel (N = 93) was constructed and genotyped with 102 SSR markers. RHMAP program was used to analyze the genotypic data. At a LOD score setting of four, 52 out of 102 SSR markers were resolved into 16 syntenic groups. LG-9 and linkage group 13 (LG-13) of the traditional linkage map were combined by WWRH mapping into one syntenic group by two-point analysis, using the RHMAP program. A WWRH map of this syntenic group was generated by maximum likelihood analysis using the RHMAP program with a general retention model. Cytogenetic analyses using monosomic and monotelodisomic hypo-aneuploid cytogenetic stocks confirmed the combination and assigned both LG-9 and LG-13 to the short arm of chromosome 17. The WWRH mapping method also corrected the chromosome location of marker BNL4053 to the long arm of chromosome 9, instead of its previous assignment to chromosome 1 in traditional linkage map. The correction was also confirmed by hypo-aneuploid marker analysis. The findings indicate that WWRH mapping will work in cotton, and that the approach complements traditional linkage mapping, as well as cytogenetic methods. We anticipate that optimized retention frequencies for cotton WWRHs will require higher rates of chromosome breakage, and that the WWRH approach will be extended to genomes of other plant taxa, at least polyploid ones.

Introduction

The cotton genus, *Gossypium*, is distributed throughout tropical and subtropical regions of the world. Of the 50 recognized natural species, most are 2n = 26 and widely regarded as diploid, but five New World species are 2n = 52 and widely regarded as tetraploid. These species are partially diploidized and seem to be *ca*. 1-2 million years old (CRONN et al. 2002; SENCHINA et al. 2003). Cytological analyses of chromosome size and meiotic affinity revealed that each 13-chromosome *Gossypium* genome could be assigned to a genomic group (A, B, C, D, E, F, G, or K), and that the groups are distributed in a geographically related manner (BEASLEY 1940, 1942; PHILLIPS and STRICKLAND 1966; EDWARDS and MIRZA 1979; ENDRIZZI et al. 1984; PERCIVAL et al. 1999). The A-genome group occurs among African-Asian species and the D-genome group occurs among species in the New World and Hawaii. Moreover, all of 52-chromosome species are disomic (KIMBER 1961), have an AD-genome composition, and can be hybridized with each other to form more or less fertile hybrid progenies. The level of *intra*-specific polymorphism for molecular markers is relatively low within the two cultivated tetraploids, *G. hirsutum* and *G. barbadense*, whereas rates of *inter*-specific polymorphism are much higher. Thus, interspecific hybridization has been used in *Gossypium* to allow for larger numbers of polymorphic loci and thus denser maps (REINISCH et al.1994; YU et al. 1998; ZHANG et al. 2002; MEI et al. 2003).

Genome maps offer extensive advantages for many sorts of genetic endeavor, such as integrative genomics, map-based gene cloning, and marker-assisted molecular breeding. Unfortunately, public maps of the cotton genome are relatively immature as compared to major crops such as rice (*Oryza sativa* L., 2n = 2x = 24) (GOFF et al. 2002; YU et al. 2002), corn (*Zea maize* L., 2n = 2x = 20) (http://www.maizemap.org), and wheat (*Triticum aestivum* L., 2n = 6x = 42) (http://wheat.pw.usda.gov /ggpages/maps.shtml#wheat). In available public linkage maps of tetraploid cotton, the number of linkage groups exceeds the gametic chromosome number (26), and numerous linkage groups have not been assigned to chromosomes (http://demeter.bio.bnl.gov /acecot.html; REINISCH et al. 1994; SHAPPLEY et al. 1998; YU et al. 1998; YU and KOHEL, unpublished data; ULLOA and MEREDITH 2000; ZHANG et al. 2002; MEI et al. 2003). Common identities have not been established between many linkage groups from different maps, and a comprehensive nomenclature is yet to be established for cotton linkage groups. However, some linkage groups have been identified across laboratories based on interspecific hypo-aneuploids. Among the factors that have hindered cotton genome map development are [1] nonrandomness of crossover distribution, [2] very high rates of recombination, [3] reliance on genome-wide marker development strategies, [4] incomplete genome coverage with hypo-aneuploids suited to physical mapping, and [5] under-utilization of molecular cytogenetic physical mapping.

The distribution of crossover and recombination are nonrandom, and both recombination hot spots and cold spots exist in many if not all higher eukaryotes (reviewed in PUCHTA

and HOHN 1996; COPENHAVER et al. 1998; GERTON et al. 2000; reviewed in PETES 2001; YU et al. 2001; ISLAM-FARIDI et al. 2002). The nonrandomness is clearly indicated by the virtual absence of cotton metaphase I bivalent configurations with chiasmata near the centromeres. Although molecular data on crossover distribution in cotton are limited, nonrandomness is implicit. For example, the A subgenomes of *G. hirsutum* and *G. barbadense* are almost twice as large as the D subgenomes (SKOVSTED 1934; BEASELEY 1942), but linkage map lengths of homeologues are similar (REINISCH et al. 1994).

Non-randomness of crossover distribution entails regions of very high or very low recombination frequency. High rates of recombination in *G. hirsutum* and other *Gossypium* species were first documented by cytological analyses of meiotic pairing in species, interspecific hybrids, and *G. hirsutum* chromosome aberration heterozygotes (SKOVSTED 1934; BEASLEY 1942). All chromosomes of *G. hirsutum* are metacentric or submetacentric, and metaphase I analyses revealed ring bivalents as the predominant meiotic configuration. The initial numerical estimates of cotton genome map length were obtained from intraspecific metaphase I chiasmata region analysis of *G. hirsutum* aberration heterozygotes, collective results of which indicated a total linkage map length of more than 4500 cM (MENZEL et al. 1985; STELLY 1993). A similar estimate was obtained from interspecific linkage analysis of molecular markers segregating amongst *G. hirsutum* x *G. barbadense* F_2 hybrids, from which the LOD-3 linkage map totaled *ca.* 4675 cM (REINISH et al. 1994).

The map estimates indicate that an average cotton chromosome arm participates in about two homologous crossovers per meiosis, and is thus about 100 cM in length. Given that the genome of *G. hirsutum* contains about 3161 Mbp (BENNETT AND LEITCH 2003), chromosome arms must average *ca.* 61 Mbp, and recombinant segments average *ca.* 30 Mbp per meiosis. In regions subject to high rates of recombination, a larger number of polymorphic markers is required to establish statistically robust linkage, unless closure is achieved by an alternative method, e.g., physical mapping. In regions low in recombination, it may be most efficient to use physical mapping approach to establish linear order. In linkage maps, low-recombination regions occur as segments of relatively high marker density and incomplete resolution. Such regions are seen in many higher density maps, including some linkage groups of cotton (REINISCH et al. 1994; ZHANG et al. 2002, MEI et al. 2003). In linkage maps constructed by linkage analysis of hybrid progenies, low-recombination regions can also arise due to non-recovery of recombinants. Most public cotton genome maps and mapping resources have relied on the use of interspecific crosses between *G. barbadense* and *G. hirsutum* to achieve high rates of polymorphism. These run a predictable but non-quantifiable risk of inversions, distortions and other factors that can reduce or compromise the effectiveness of linkage analysis alone for genome mapping. As is typical for wide crosses, distorted marker segregation is commonly observed in linkage mapping populations from *Gossypium* interspecific hybridization (REINISCH et al. 1994; ZHANG et al. 2002; MEI et al. 2003). Moreover, distorted marker segregation was also observed in an intraspecific *G. hirsutum* mapping population (SHAPPLEY et al. 1998).

The difficulty of reducing the number of cotton linkage groups to the gametic number reflects a need for physical mapping. Where hypo-aneuploid cytogenetic stocks are lacking, most efforts to distinguish and identify high-recombination regions have relied on large-scale marker development strategies that are genome-wide, not targeted to the specific segment. Molecular cytogenetic solutions have not been used, even though cotton bacterial artificial chromosome (BAC) clones were among the first BACs used for fluorescent in situ hybridization (FISH) in plants or animals (HANSON et al. 1995). Thus, the need for relatively few markers in certain high recombination regions of cotton continues to be addressed by large-scale marker development on a genome-wide scale.

The aforementioned data suggest the superficially ironic conclusion that resolution and integration of cotton genome maps can be improved through both reduced and increased recombination. Reduced recombination is needed in regions with naturally high rates of crossing over, but increased recombination is needed in those with naturally low rates of crossing over. In this report, we examine the feasibility of addressing both of these needs through the use of radiation hybrids.

Goss and Harris (1975) first used radiation-induced chromosome rearrangement to map genes on human chromosome X. After lethal X-ray irradiation of human cell lines, fragmented human X chromosome were rescued by rodent cells by means of cell fusions. In contrast to natural recombination, radiation-induced recombination frequencies and map resolution can be modulated by modifying radiation doses.

However, this technique for physical mapping was not widely employed until Cox et al. (1990) used radiation hybrids for the construction of a high resolution map of human chromosome 21 and termed this technology as radiation hybrid (RH) mapping. RH mapping not only increased the recombination events, but also offered the advantages of very high rates of polymorphism between donor and recipient cell lines. Thus, it became possible to use markers monomorphic within the donor species for mapping (COX et al. 1990).

A major limitation of the RH mapping method by Cox et al. (1990), however, is that it maps only one chromosome at a time. Given large population sizes about 200-300 RH cell lines for relatively high resolution, it is difficult to generate a high-resolution map of organisms with large genomes (WALTER et al. 1994; RIERA-LIZARAZU et al. 2000). To overcome this limitation, Walter et al. (1994) developed a radiation hybrid mapping method, termed whole-genome radiation hybrid (WGRH) mapping. In WGRH mapping, whole-genome radiation hybrids are generated when the donor material is obtained from a diploid cell line of the donor species, rather than from a somatic cell hybrid single-chromosome addition line (COX et al. 1990; WALTER et al. 1994). The WGRH mapping is vastly superior to RH mapping because it allows all chromosomes to be mapped using a single radiation hybrid panel (WALTER et al. 1994). The WGRH mapping approach has been rapidly assimilated into genome mapping efforts for humans and certain animal species (MCCARTHY 1996; SCHMITT et al. 1996; MCCARTHY et al. 1997; WOMACK et al. 1997; KWOK et al. 1998, 1999; PRIAT et al. 1998; YERLE et al. 1998; GEISLER et al. 1999;

HAWKEN et al. 1999; MURPHY et al. 1999; VIGNAUX et al. 1999; WATANABE et al. 1999; KIGUWA et al. 2000; REXROAD et al. 2000; OLIVIER et al. 2001).

In plants, radiation treatments have been used mainly for inducing mutation (DRISCOLL et al. 1963; KOHEL 1973; ISLAM et al. 1981; RILEY et al. 1984; reviewed in AHLOOWALIA and MALUSZYNSKI 2001). Radiation treatments have been used to mutate genes, to generate translocation lines, and aneuploidies in cotton (KOHEL 1973; ENDRIZZI et al. 1985; STELLY, unpublished data). RH mapping of an oat-maize addition line was recently used to map maize chromosome 9 (RIERA-LIZARAZU et al. 2000), i.e., where the scope of mapping for each RH panel is relegated to a single chromosome, similar to the RH method of Cox et al. (1990). Recently, Wardrop et al. (2002) reported *in vitro* WGRH cell line panels developed by incorporation of barley (*Hordeum vulgare*) genome fragments into tobacco (*Nicotiana tabacum*) protoplasts. The difficulty of finding appropriate recipient cell lines to rescue irradiation-fragmented plant chromosomes has been a major obstacle in the application of RH or WGRH in mapping plant species. To circumvent this limitation, we instead used a different but related species to rescue irradiated chromosomal segments. Rather than introduce fragmented chromosomes *in vitro*, and dealing with extensively mosaic cell populations, as in traditional RH or WGRH mapping methods, we used the genome of one species to rescue irradiated chromosomal segments of another species within the same genus through wide crossing. We termed this modified WGRH mapping approach as wide-cross whole-genome radiation hybrid (WWRH) mapping. Tetraploid cotton species were

employed to test the feasibility of the WWRH mapping method because they can tolerate aneuploidy. In this article, we present initial research results on WWRH in cotton, which indicate it to be a promising mapping approach that will help improve the overall cotton genome map.

Materials and methods

Plant materials: One accession from each of two tetraploid cotton species were chosen as parents to generate a WWRH panel: the female parent 3-79 is a doubled haploid line of *Gossypium barbadense* L. (2n = 4x = 52) with $(AD)_2$ genomes, and pollen parent TM-1 is a highly inbred line of *Gossypium hirsutum* L. (2n = 4x = 52) with $(AD)_1$ genomes. They were selected as parents because they have been used extensively for linkage mapping (http://demeter.bio.bnl.gov/acecot.html; YU et al. 1998; YU and KOHEL, unpublished data), tolerate aneuploidy; and are homozygous or largely homozygous. Relatively high molecular marker polymorphism exists between them; and both are cultivated forms of cotton.

Radiation treatment: TM-1 flowers at anthesis were irradiated with γ-rays at the Texas A&M University Nuclear Science Center (NSC) or the College of Veterinary Medicine (CVM), then used to pollinate 3-79 flowers emasculated the day before. Four radiation dosages, 1.5 krads and 5 krads at NSC, and 15 krads and 30 krads at CVM, were used to irradiate TM-1 flowers. For dosages of 1.5 and 5 krads, about 150 cross-pollinations

were made, respectively, and for dosages 15 and 30 krads, about 80, respectively. The number of F_1 seed, F_1 seed germination frequency, and F_1 plant chromosome deletion types and deletion frequencies were surveyed as described below. The data were used to select which might be the ‘best’ dosage for constructing a WWRH mapping panel, where desirable features include relatively high frequencies of segmental deletions and hypo-aneuploidy, and sufficient recovery of viable F_1 seed and F_1 plants.

Radiation hybrid genotyping: Thirty-three SSR markers from four chromosomes and one linkage group were used to genotype and characterize the pilot WWRH panels. Up to 22 WWRH plants were included in the radiation dosage pilot-screening population for each of the dosages. A WWRH mapping panel composed of 93 RH plants was constructed for the selected radiation dosage (5 krads) and was genotyped with more SSR markers. Experimental controls included the two parents, 3-79 and TM-1, as well as non-irradiated 3-79 x TM-1 F_1 hybrids.

DNA samples were extracted from young leaf tissues of about two-month old plants. SSR primers were purchased from Research Genetics Inc. Each PCR reaction included 100 ng genomic DNA, 0.2 mM dNTPs, 3.0 mM $MgCl_2$, 0.04 μM forward primer, 0.04 μM reverse primer and 0.5 unit of Taq polymerase in 15 μl total reaction volume. PCR reaction was performed as follows: 95 °C for 2 min; 40 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min; 72 °C for 7 min; and 4 °C for holding. PCR products were resolved in 4% agarose gels. WWRHs missing the expected TM-1 PCR amplification

products, i.e., SSR markers, were identified as deletion lines (Figure 1). All 5_{krad} WWRHs (101 in total) were genotyped twice for given SSR markers from LG-9 (YU and KOHEL, unpublished data). In the second round of genotyping, all conditions were the same as that mentioned above, except that the total PCR reaction volume was increased to 30 µl. All other SSR markers were genotyped only once on the constructed 5_{krad} WWRH mapping panel which has 93 WWRHs.

Statistical analysis: The presence/absence (+/-) of each marker was scored for each WWRH, and a question mark (?) was assigned to a radiation hybrid when its band pattern was not sure, then marker retention frequencies were calculated in the respective WWRH panel. The retention frequency for a given marker was calculated simply as the ratio of number of WWRH plants carrying the marker band to total number of WWRH plants unambiguously screened in that panel. The radiation dosage deemed to offer the best combination of high plant viability and high marker deletion was chosen for more detailed characterization of a given linkage group. For that purpose, we used SSR markers of LG-9. Chi-square tests were carried out to determine if SSR marker retention frequency was independent of their location in LG-9. LG-9 was arbitrarily separated into eight segments each represented by one SSR marker on it. Marker retention frequency and pattern were used to represent the corresponding chromosomal segment retention frequency and pattern. Markers that had been co-localized in the linkage map were logically ordered according to marker retention patterns in the WWRH panel and the

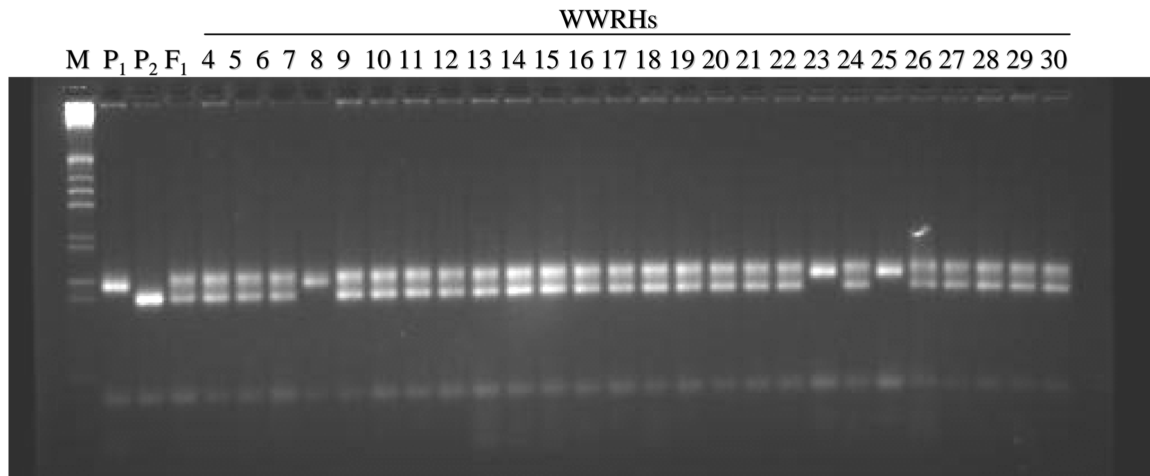

Figure 1: Detection of WWRH deletion lines. PCR products of SSR marker BNL1066 were resolved in a 4% agarose gel. Lane 1 is molecular ladder (M); lane 2, *G. barbadense* line 3-79 (P_1); lane 3, *G. hirsutum* line TM-1 (P_2); lane 4, normal F_1 hybrid from 3-79 x TM-1; lanes 5 to 30, WWRHs. All WWRHs (lanes 5-30) had bands from female parent 3-79. Symbols "+" and "-" indicate the present and absent band patterns of PCR products from the pollen parent, respectively. WWRHs in lanes 8, 23 and 25 indicated missing bands of PCR products from TM-1 and were identified as WWRH deletion lines.

minimum obligate breaks approach. Multiple-point radiation hybrid mapping program RHMAP version 2.01 (BOEHNKE et al. 1995) was also used to generate a WWRH map (see next).

RHMAP analysis: Genotypic data from 102 SSR markers with good genotyping quality, that is, with fewer uncertain genotypes, were employed to run RHMAP program to test the feasibility of generating a WWRH map. Because only 60 markers could be used in running the RHMAP program at one time, several rounds of two-point RHMAP analyses were carried out using genotypic data from sixty SSR markers (out of 102) at one time to identify syntenic groups. The minimum LOD score of four was set for a significant syntenic group. Based on two-point analysis, we chose the syntenic group containing the most marker loci for further analysis. This group of marker loci was subjected to maximum likelihood analysis using the RHMAP program to construct a WWRH map.

Integration mapping and cytogenetic aneuploid analysis: The WWRH syntenic groups generated from RHMAP analyses were compared and contrasted with those from conventionally developed molecular marker linkage maps (http://demeter.bio.bnl.gov/acecot.html; YU et al. 1998; YU and KOHEL, unpublished data). In current cotton linkage maps, not all linkage groups have been associated with chromosomes and the number of linkage groups exceeds that of gametic chromosome number. When we associated an SSR marker from a linkage group with SSR marker(s)

from a syntenic group in our WWRH map, the linkage group was assigned to that syntenic group. When two or more SSR markers from different linkage groups were found syntenic in our WWRH map, the corresponding linkage groups were combined. Hypo-aneuploid interspecific hybrids were used to confirm the assignments of linkage groups to syntenic groups, and to conduct synteny analysis, i. e., to detect the linkage groups originating from the same chromosome. The development and application of similar or identical stocks for chromosome assignments have been described previously (REINISCH et al. 1994).

Results

Dosage effect analysis: The number and quality of seed resulting from cross-pollination with γ-irradiated pollen differed markedly across the radiation dosages. Virtually all F_1 seed formed after the 1.5-krad and 5-krad treatments were normal in appearance, whereas no viable seed were obtained from the higher dosages. No seed were recovered after pollinating with pollen that received the 15-krad treatment, while four small seed were formed after pollinating with pollen that received the 30-krad treatment, but all were motes.

Pilot-screening samples of 25 1.5-krad and 26 5-krad WWRH F_1 seed were germinated. Germination percentages were similar -- 25/25 for the former and 24 / 26 for the latter. Of the two un-germinated 5-krad RH F_1 seed, one was hollow with nearly black seed

coat, the other was not hollow but small, about two-thirds of normal size. All of the other seed planted were normal in appearance.

Considering the size of our electrophoresis apparatus, preliminary genotypic evaluation was conducted on 44 WWRH F_1 plants, 22 randomly selected from each treatment of 1.5 krads and 5 krads, respectively. The plants were genotyped with 33 SSR markers from one linkage group and four chromosomes, of which eight, six, seven, six, and six markers were previously mapped to LG-9, and chromosomes 10, 12, 18 and 26, respectively (YU and KOHEL, unpublished data). The incidence of deletions was considerably higher and the types of deletions more diverse among 5-krad than 1.5-krad WWRH plants. Among the 22 WWRHs from 1.5-krad γ-ray treatment, only two (9.1%) were identified as deletion lines. One plant lacked all six chromosome 12 markers, which indicates possible monosomy, and the other lacked just a terminal marker from the end of chromosome 10, most likely due to a single break and loss of a terminal segment (Figure 2). Marker retention frequency among the 1.5-krad plants was thus quite high, from 95.5 to 100% for individual loci, and averaged 98.4%. In contrast, among the 22 WWRHs from 5-krad γ-ray treatment, eleven (50.0%) lacked one or more of the markers from LG-9 and/or chromosomes 10, 12 and 26, but no deletion lines observed involved chromosome 18. Four different types of deletions were detected (Figure 2). These included deletions of marker(s) at one end of a linkage group (one-end deletion, e.g., from a single break), deletions of markers at both ends of a linkage group (two-end deletion, e.g., from two breaks in opposite arms), deletions of interstitial

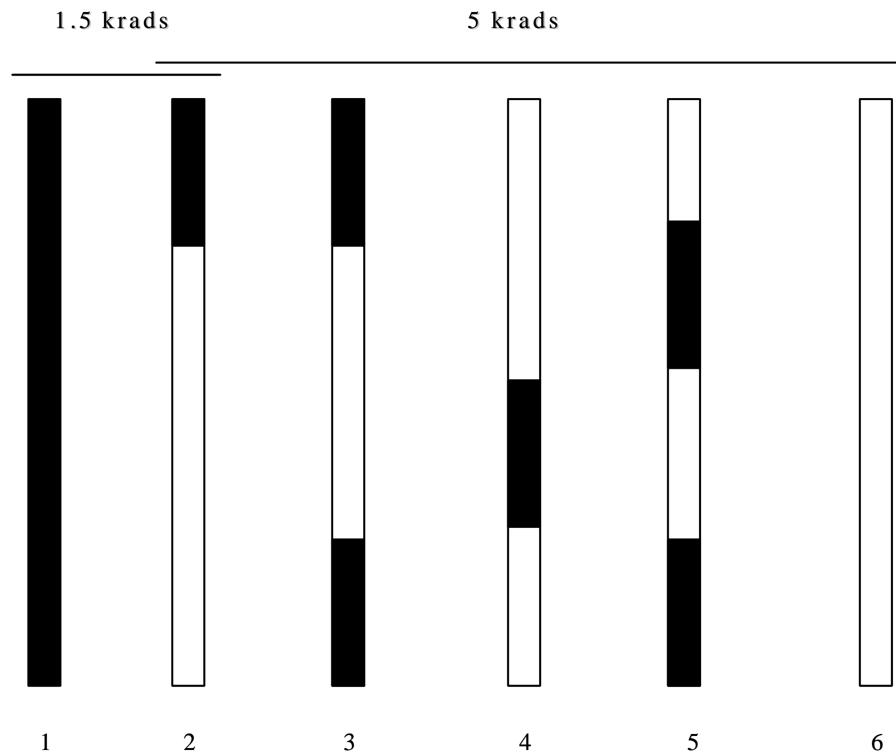

Figure 2: Deletion classes observed in 1.5-krad and 5-krad panels. Each long bar represents a chromosome or linkage group. Short black bar represents the location where a chromosomal segment was deleted from the corresponding chromosome or linkage group. 1 = whole deletion, 2 = one-end deletion, 3 = two-end deletion, 4 = internal deletion, and 5 = one-end plus internal deletion, 6 = no deletion.

marker(s) only (internal deletion, e.g., from two breaks within an arm, followed by fusion or translocation(s)), and a more complex pattern (one-end plus internal deletion, e.g., from three breaks, one leading to a terminal deletion, and the other two occurring within an arm, leading to an interstitial deletion). For individual marker loci, retention frequency ranged from 77.3 to 100%, and the average across all loci was 93.0%.

These findings strongly suggest that the 5-krad γ-ray treatment would be far more efficient than 1.5-krad γ-ray treatment for producing a WWRH panel. They also suggested that a more efficient optimal radiation treatment dosage might be established between 5 and 15 krads of γ-rays.

SSR analysis of LG-9: Ninety-one additional WWRH seed from 5-krad pollen irradiation were germinated, from which 79 additional WWRH F_1 plants generated. Of the other twelve seed, nine were hollow (lacked a developed embryo), and three underwent radical elongation but died after initial seedling development. The 79 plants were combined with the pilot-screening population of 22 5-krad WWRH plants to construct a 5_{krad}-WWRH panel of 101 individuals. LG-9 was chosen to evaluate the WWRH panel because eight available SSR markers (Table 1) were relatively evenly distributed along the linkage group (Figure 3; YU and KOHEL, unpublished data). Two of them, BNL0625 and BNL2805, co-segregated in the linkage map population (http://demeter.bio.bnl.gov/acecot.html; YU and KOHEL, unpublished data).

TABLE 1

Eight SSR markers of linkage group 9 tested in WWRH lines

Locus	Primers	Product size (bp)
BNL0625	AGAGAGGGGGGAAAAGTTCA GCCAGGCATGGTTTCTATGT	250
BNL0836	ATCTTGTTGATTTTCTGACTACAGG CAGACATTCCCCTTCCTTGA	190
BNL1066	ACATTTCCACCCAAGTCCAA ACTCTATGCCGCCTCTCGTA	130
BNL2632	CGTGTCTCCAGACCAACAAA GGGAGTTGAAGCCGACATAA	250
BNL2805	AGTTTGGAATTACAATAAATGTACTCG CCAAGGTCGGTCGGTTACTA	240
BNL3254	CACACAGTGTCCTTTGGGTG AGCCTCAAAGGCCAAAAGTT	130
BNL3592	GTTCTAGTCTCTTTCTTTTATGGGC TTGATTGAGATGCCAATGGA	200
BNL4094	ATGCTGCGGAGTCGATATCT AAATTGATTTCATGCCGGAG	170

To characterize the deletion patterns, LG-9 was arbitrarily separated into eight parts in the dissection map in Figure 3. Each part was represented by one of the eight genotyped SSR markers in LG-9 regardless of their distances along the linkage group. Using the eight LG-9 SSR markers, 20 of the 101 WWRH plants were identified as deletion lines with ten different deletion genotypes (Figure 3, Table 2). Individual deletion genotypes were observed in up to five WWRH individuals (Figure 3, Table 2). For individual SSR marker loci, the retention frequencies ranged from 87 to 94% with an average of 89.5% (Table 3). Chi-square tests indicated that marker retention frequencies did not depart significantly from the hypothesis that marker retention was independent of marker location on LG-9 (Table 3).

Whereas SSR markers BNL0625 and BNL2805 co-segregated in the traditional linkage mapping population, they were separated in two WWRH deletion lines, GH6550 and GH6707. If the order of BNL2632, BNL0625, BNL2805 and BNL3592 along LG-9 is BNL2632-BNL0625-BNL2805-BNL3592, at least three breaks are required for GH6550 and GH6707 to form their genotypes in Table 2. Alternatively, if the order is BNL2632-BNL2805-BNL0625-BNL3592, only one break would be enough to form their genotypes in Table 2 and Figure 3. Out of 101 irradiated gametes represented in the WWRH panel, the likelihood of two gametes each with one break between BNL2805-BNL0625 would be much higher than the likelihood two gametes each with three breaks between BNL2632-BNL0625, BNL0625-BNL2805 and BNL2805-BNL3592. Accordingly, the suggested locus order of BNL0625 and BNL2805 in LG-9 is

BNL2632-BNL2805-BNL0625-BNL3592 (Figure 3). This order based on probability and minimum obligated breakages was confirmed by the maximum likelihood RHMAP analysis with general retention probability model (COX et al. 1990; BOEHNKE et al. 1995) using the 5_{krad}-WWRH mapping panel (Figures 3 and 6). The distance between BNL0625 and BNL2805 was 14.5 $centiRay_{5krad}$ (Figure 6). The total WWRH map length of LG-9 was 118.7 $centiRay_{5krad}$. The marker distance correlation coefficient between the traditional linkage map (YU and KOHEL, unpublished data) and the WWRH map was 0.49.

RHMAP analysis: For genotyping convenience, 93 WWRHs were randomly selected from the 101 5-krad WWRHs to form the 5-krad WWRH mapping panel. Good quality genotypic data with fewer than eight (< 8.6%) WWRHs having uncertain genotypes for a given marker, from 102 SSR markers were employed to run two-point RHMAP analyses. Syntenic groups were identified at three LOD score levels (LOD = 4, 6 and 8, respectively), as listed in Table 4. Fifty-two out of 102 SSR markers were found syntenic with one or more SSR markers at LOD-4. We believed that the number of markers tested affected the number of markers that were statistically detectable as syntenic with other markers. The more markers tested, the more saturated the genome will be, and the more markers will be detected as syntenic with other markers. In some cases, one linkage group was separated into different syntenic groups in the WWRH map. For example, LG-9 was separated into two syntenic groups. On the other hand, parts of different linkage groups were mapped into a single syntenic group, e.g., part of

Figure 3: Types of WWRH deletion lines based on eight LG-9 loci. Out of 101 WWRHs of 5-krad γ-ray treatment, 20 were identified to lack one or more LG-9 markers. The 20 deletion lines were grouped into 10 different types according to their patterns of SSR marker retention. The number of individuals in each type is listed below the genotype pattern. At the left is a traditional linkage map of LG-9, immediately to its right is a WWRH map on LG-9 based on 93 WWRHs in the 5_{krad} mapping panel. Whereas SSR markers BNL0625 and BNL2805 co-segregated in the linkage mapping population, they were resolved in the WWRH panel (see text). The dissection map shows LG-9 dissected into eight arbitrarily equal parts, each represented by one SSR marker. Solid bar = deleted chromosomal segment; empty bar = undeleted chromosomal segment.

TABLE 2

Genotypes of WWRHs with deletion(s) in linkage group 9

RH Lines	BNL4094	BNL3254	BNL2632	BNL0625	BNL2805	BNL3592	BNL1066	BNL0836
GH6541	+	?	+	+	?	-	-	-
GH6545	-	-	-	-	-	+	+	+
GH6550	-	-	-	+	-	+	+	+
GH6552	-	-	-	-	-	+	+	+
GH6556	+	+	+	+	+	-	-	-
GH6557	-	-	-	-	-	+	+	+
GH6558	-	-	-	+	+	-	-	-
GH6663	-	-	-	-	-	+	+	+
GH6678	+	+	+	+	+	+	-	-
GH6691	-	-	-	+	+	+	+	+
GH6692	-	-	-	-	-	-	-	-
GH6693	?	+	+	+	+	+	-	-
GH6699	+	+	+	+	+	-	-	-
GH6670	+	+	+	+	+	-	-	-
GH6702	+	+	+	+	+	-	-	-
GH6707	-	-	-	+	-	+	+	+
GH6713	-	-	-	+	+	+	?	+
GH6722	+	+	-	+	+	-	-	-
GH6724	-	-	-	-	-	+	-	-
GH6725	-	?	-	+	+	+	-	-

- = deleted for correspondent SSR marker
+ = heterozygous for correspondent SSR marker, i.e., undeleted
? = undecided genotype

TABLE 3

Linkage group 9 SSR marker retention frequencies and c^2 test

Locus	Typed[a] (Heterozygotes : Deletions)	Retention frequency
BNL0625	100 (94 : 6)	0.9400
BNL0836	99 (87 : 12)	0.8788
BNL1066	92 (80 : 12)	0.8696
BNL2632	100 (87 : 13)	0.8700
BNL2805	99 (91 : 8)	0.9192
BNL3254	95 (84 : 11)	0.8842
BNL3592	99 (91 : 8)	0.9192
BNL4094	99 (87 : 12)	0.8788
Average		0.8950
χ^2		5.37 ($\chi^2_{0.05}$ = 14.07; v = 7)

a = number of WWRH lines unambiguously scored as deletion and non-deletion lines, followed by number of non-deletion lines and number of deletion lines in parentheses.

TABLE 4

Linkage groups detected by two-point RHMAP analyses at three LOD-score levels

LOD score	SSR markers found to be syntenic
4	1604, 3065
	1667(lg11), 3888(lg11)
	2448(lg24), 3992(lg24)
	2553(lg17), 3646(lg17)
	2570(ch20), 3838(ch20)
	2646, 4082(lg11)
	2986(lg20), 3008(lg20)
	3103(ch25), 3264(ch25)
	836(lg9), 1066(lg9), 3592(lg9)
	1672, 3140(lg21), 3511(lg21)
	1705(lg26), 3449(lg26), 3976(lg26)
	2847(ch9), 3779(ch9), **4053**(lg11)
	3255(ch5), 3474, 3792(g12)
	1317, 1350, 3345(lg11), 3902(lg11)
	1161(ch10), 1665(ch10), 2960(ch10), 3563(ch10), 3895(ch10)
	625(lg9), 1034(lg13), 1151(lg13), 1404(lg13), 1681(lg13), 2632(lg9), 2805(lg9), 3254(lg9), 3411(lg13), 3431(lg13), 3442(lg13), 4094(lg9)
6	1667, 3888
	3449, 3976
	1350, 3345, 3902
	836, 1066, 3592
	1161, 1665, 2960, 3563, 3895
	625, 1034, 1151, 1404, 1681, 2632, 2805, 3254, 3411, 3431, 3442, 4094
8	836, 1066
	1667, 3888
	1161,1665, 2960, 3563, 3895
	1034, 1151, 1404, 1681, 2632, 2805, 3254, 3411, 3431, 3442, 4094

Notes: The prefix BNL was omitted from the identification of each SSR marker. Marker locations if available in traditional linkage map are listed in parentheses at LOD-4. lg = linkage group; ch = chromosome

LG-9 and part of LG-13 were mapped into the same syntenic group. No markers known to be located in different chromosomes were found mapped into same syntenic group. However, one linkage group, linkage group 11 (LG-11), was found associated with two chromosomes, chromosome 1 and chromosome 9 (see next). Slight marker order differences were noticed between the linkage maps of LG-9 by Yu and Kohel (Figure 3) and Burr (http://demeter.bio.bnl.gov/acecot.html). Maximum likelihood RHMAP analysis under the general retention probability model (COX et al. 1990; BOEHNKE et al. 1995) of the 93 WWRHs 5_{krad} mapping panel gave the most likely order as that in the map from Yu and Kohel.

Integration mapping and cytogenetic stock analysis: According to our results, LG-9 and LG-13 involve markers from the same chromosome because several markers from each linkage group were mapped into the same syntenic group at LOD = 8 in our 5_{krad} WWRH panel. Cytogenetic aneuploid stocks were employed to identify the chromosomes associated with those two linkage groups and to test the WWRH-based deduction regarding their synteny. Although the collection of cotton hypo-aneuploid cytogenetic stocks is still incomplete, we screened interspecific monosomic chromosome substitution stocks for chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 23, and 25 with SSR markers BNL1066 from LG-9 and BNL3442 from LG-13. Absence of both markers from H17 indicated that both markers are associated with chromosome 17, and that chromosome 17 harbors both LG-9 and LG-13 (Figure 4A and 4B). Further analyses were conducted with the monotelodisomic "Te17sh", which contains a normal

chromosome 17 and a telosome for the short arm of chromosome 17. The presence of both markers indicated that this telosome contains both markers and most or all of LG-9 and LG-13 (Figure 5A and 5B). A WWRH map of the syntenic group by combining LG-9 and LG-13 was generated from the maximum likelihood RHMAP analysis with the general retention model (Figure 6). It was noticed that the distance among markers from LG-13 was zero in the WWRH map. We checked the genotyping data. The average marker retention frequency for those markers on LG-13 was 92.4% with a range from 90.0% to 96.5% calculated from 93 WWRHs, whereas markers from LG-9 had an average of 89.5% with a range from 87.0% to 94.0% calculated from 101 WWRHs (Table 3). When all markers from both LG-9 and LG-13 were used, the Chi-square test ($\chi^2 = 0.08 < \chi^2_{0.05} = 31.32$, v = 14) indicated that marker retention frequency was still independent of SSR markers on the newly combined syntenic group. However, we found significant differences in deletion types. For LG-13, all 13 of the deletions were of one deletion type (if not considering uncertain genotypes), whereas for LG-9, there were 10 different deletion types among 20 deletions.

WWRH results indicated that LG-11 (YU and KOHEL, unpublished data) should be assigned to chromosome 9 because marker BNL4053 from LG-11 was syntenic to markers BNL3779 and BNL2847 from chromosome 9 at LOD-4. To test this inference, we examined the interspecific F_1 hybrid stocks monosomic for Chromosome 9 (H9) and monotelodisomic for chromosome 9 long arm (Te9Lo). BNL4053 was thereby

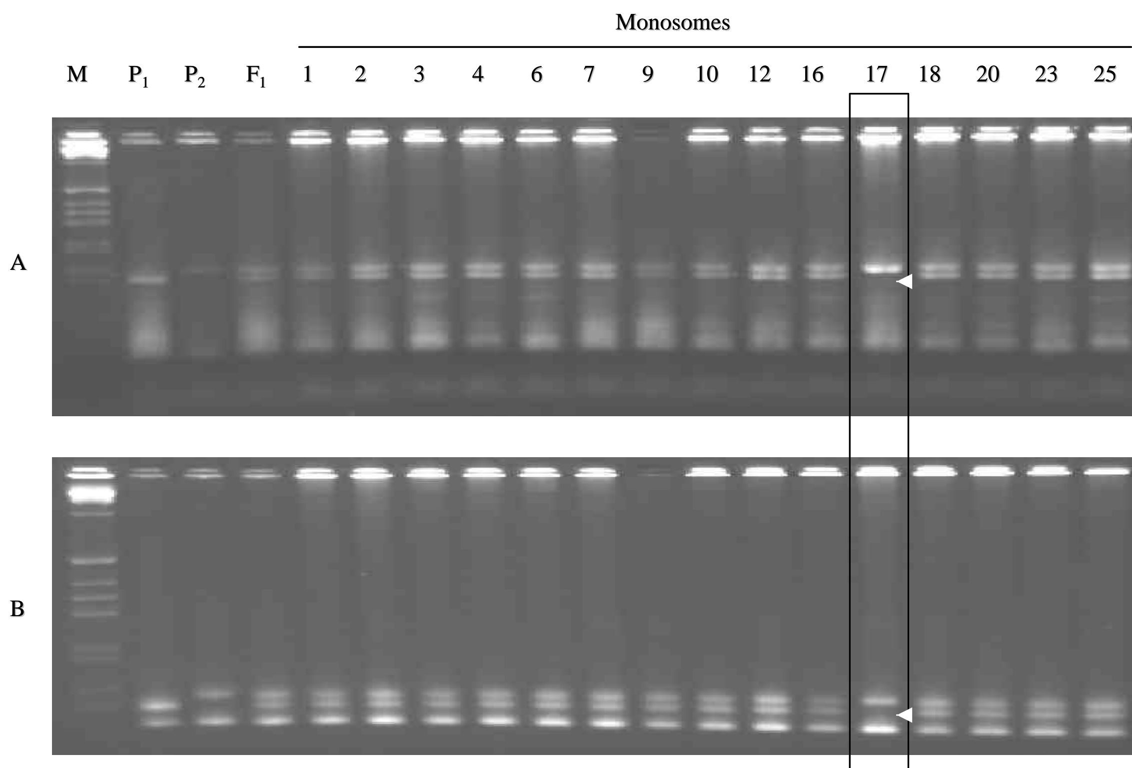

Figure 4: Cytogenetic confirmation of the assignment of LG-9 and LG-13 to chromosome 17 using available monosomic chromosome substitution lines in cotton. Lanes 1 through 19 are molecular ladder (M), TM-1 (P_1), 3-79 (P_2), F_1 (3-79 x TM1) and interspecific substitution stocks monosomic for chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 23, and 25, respectively. Panel A: Gel picture for marker BNL1066 from LG-9. Panel B: Gel picture for marker BNL3442 from LG-13. Monosomic substitution line for chromosome 17 is boxed, and the missing bands are denoted by arrowheads for both markers.

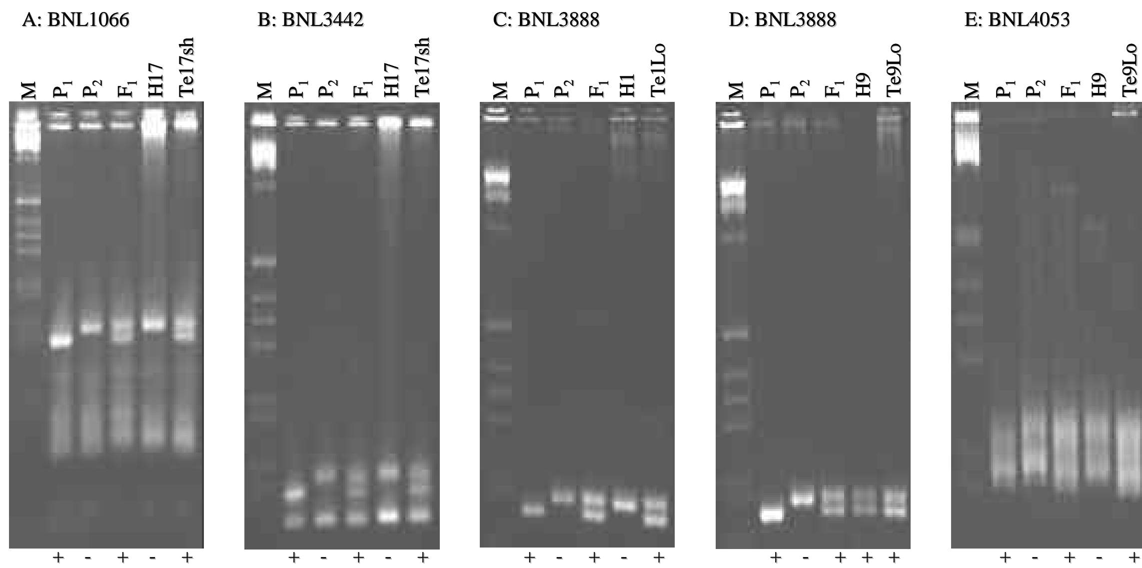

Figure 5: Cytogenetic confirmation, using corresponding monosomes and ditelosomes, of the assignments of (1) LG-9 and LG-13 to the short arm of chromosome 17 (A and B), (2) LG-11 to chromosome 1 (C) rather than to chromosome 9 (D), and (3) marker BNL4053 on chromosome 9 long arm (E). Lane 1 through lane 3 are molecular ladder (M), TM-1 (P_1), 3-79 (P_2), F_1 (3-79 x TM-1) respectively. Lane 4 = monosomic substitution lines for chromosome 17 in panels A and B, chromosome 1 in panel C, chromosome 9 in panels D and E, respectively. Lane 5 = monotelodisomic lines: Te17sh in panels A and B, Te1Lo in panel C, and Te9Lo in panels E and F, respectively, sh = short arm, Lo = long arm. + = target band present, - = target band absent (see text).

associated with chromosome 9, specifically with the long arm of chromosome 9 (Figure 5E). However, other markers from LG-11 could not be assigned to chromosome 9 according to additional cytogenetic analyses. For example, marker BNL3888 was actually associated with chromosome 1 (Figure 5C) rather than chromosome 9 (Figure 5D). Examining LG-11, it was found that marker BNL4053 was mapped at one end of the linkage group, the distance between it and the next marker BNL2921 was 44.4 cM at a LOD score of 3.46 (YU and KOHEL, unpublished data). This indicated that it was a false positive, and that marker BNL4053 was incorrectly assimilated into LG-11 in the traditional linkage map. According to our WWRH mapping and cytogenetic analyses, we concluded that the WWRH mapping method correctly located marker BNL4053 into chromosome 9 and it corrected the false positive linkage in traditional linkage mapping.

Discussion

Advantages of the WWRH mapping method: Cell line and selectable *in vitro* traits have allowed for the rapid adoption of RH and WGRH methods for mapping human and various animal genomes (MCCARTHY 1996; SCHMITT et al. 1996; MCCARTHY et al. 1997; WOMACK et al. 1997; DELOUKAS et al. 1998; KWOK et al. 1998, 1999; PRIAT et al. 1998; YERLE et al. 1998; GEISLER et al. 1999; HAWKEN et al. 1999; MURPHY et al. 1999; VIGNAUX et al. 1999; WATANABE et al. 1999; KIGUWA et al. 2000; OLIVIER et al. 2001), but analogous cell lines and technologies are generally lacking among angiosperm species. Ideally, a common or limited number of recipient genomes could serve as a

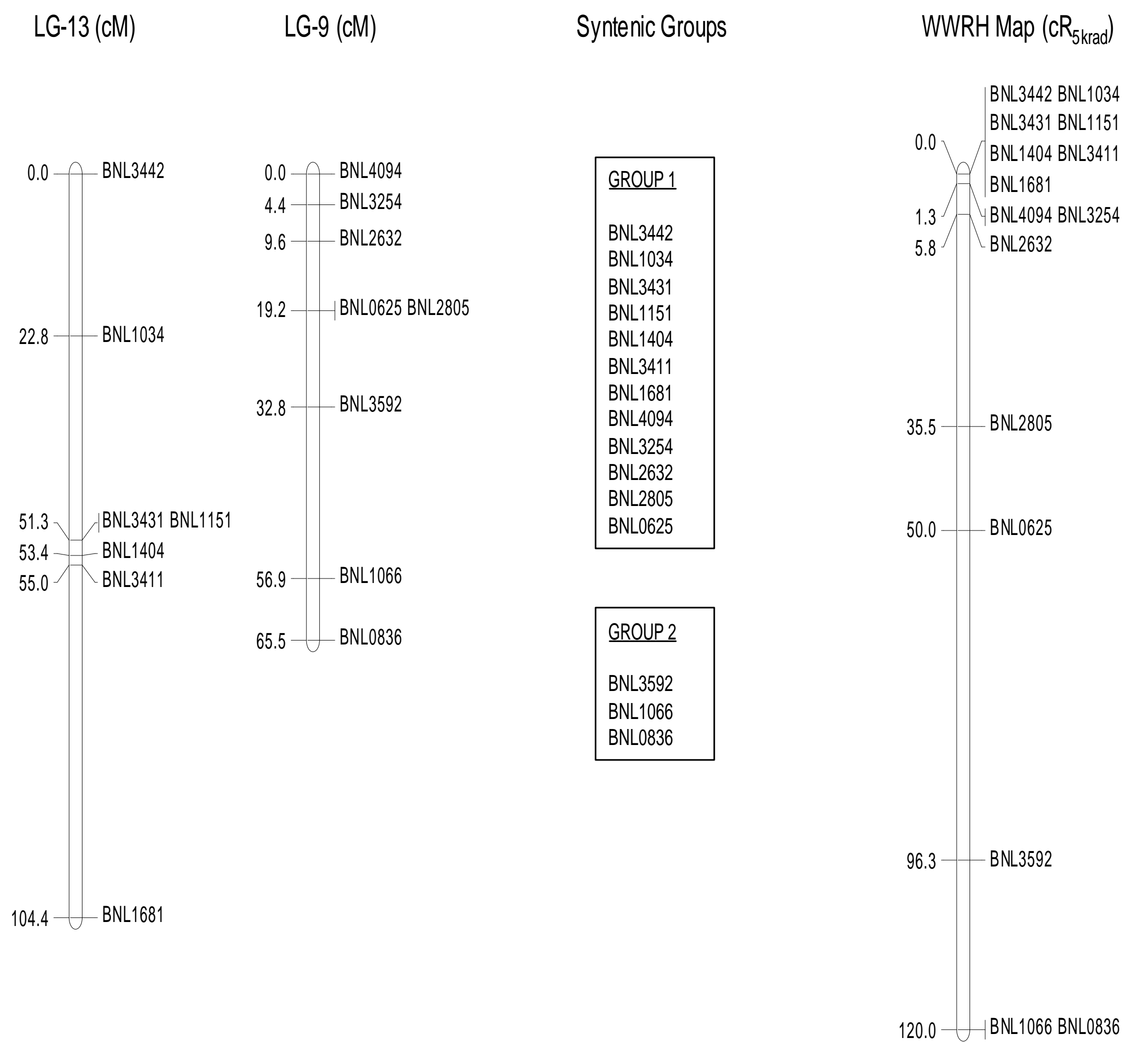

Figure 6: Traditional linkage maps of LG-9 and LG-13 and the corresponding WWRH map. The middle panel shows the syntenic groups at LOD-4 by two-point RHMAP analyses.

recipient for segmented genomes from diverse angiosperm taxa. In lieu of *in vitro* culture and fusion technologies comparable to those in animals, it's likely that plant researches will devise a number of different and largely idiosyncratic approaches. For example, there was a long history of intergeneric and interspecific hybridization among various taxa in the *Gramineae* to increase genetic diversity, to construct genetic analysis stocks, generate doubled haploids, and even to generate new species such as triticale. Several maize chromosomes were added into oat genetic background to get oat-maize addition lines (RIERA-LIZARAZU et al. 1996, 2000). Beginning with oat-maize addition line, the RH panel for maize chromosome 9 was developed (RIERA-LIZARAZU et al. 2000). In comparison with radiation hybrid mapping methods used in human, animal, barley and maize, the WWRH mapping method is quicker, simpler and less expensive, yet effective. First, WWRH is a modified WGRH mapping method that maps whole genomes via a single WWRH mapping panel, which is more efficient than RH mapping approach that requires different mapping panels to map different chromosomes. Second, the rescue process of segmented chromosomes is simple. Specifically, we used *G. barbadense* to rescue chromosomal segments of *G. hirsutum.* The irradiation-segmented donor *G. hirsutum* genomes were introduced into recipient *G. barbadense* nucleus through wide crossing. To facilitate the irradiation treatment and the introduction of the segmented DNA to the recipient cell, we used irradiated pollen followed by conventional manual cross-pollination procedures. This *in vivo* rescue process greatly simplified the rescue process as compared to those used to generate (WG)RHs *in vitro* for human, animals, and barley genomes. Third, this pollen irradiation approach describe herein is

quick and relatively inexpensive. Except for hybrid clonal crops, most plant mapping populations are constructed by hybridization, followed by development of mapping populations at F_2, backcross, and/or advanced recombinant-inbred stages. In the WWRH method, the quasi-F_1 plants are well suited for mapping without further breeding, i.e., allowing for rapid map development and alleviating the requirement for fertility in order to conduct an analysis. For plant species with long regeneration cycles, WWRH could be a quick approach to construct a mapping population and map. Fourth, WWRH might apply for more diverse parents. WWRHs, the F_1 generation, are employed as mapping panel, so WWRH panels can be constructed between parents that form hybrids, without regard to the fertility of their hybrids, allowing the use of more genetically distant parents. Using parental genotypes more distinct than *G. barbadense* and *G. hirsutum*, it will be possible to map greater proportions of markers, including those that are monomorphic in linkage mapping populations of relatively closer species. Thus, we expect that use of other parents will increase the percentage of markers that can be mapped by WWRH method, however, the percentage will expectedly fall below the percentages possible via somatic cell fusion approach in WGRH or RH mapping methods because, in our WWRH method, the parents must be closely enough related to form viable hybrid with chromosomal re-constitutions. On the other hand, the facility of working with plants in a semi-conventional manner is a clear benefit. For perennial species, such as cotton, where the plants can be vegetatively propagated and even increased, the resulting WWRH panels can be distributed and used for other sorts of analyses without worry about the panel DNA sources. In contrast to *in vitro* culture

system, there is less cause to worry about instability and chimerism of rescued chromosome fragments in the panel components. Kwok (1998) reported all chromosome segments could be retained without selection over six (zebrafish) and 25 (chicken) generations of sub-culture, respectively. However, rescued fragment stability in host cell is a concern in traditional (WG)RH mapping approaches in human and animal species.

In present method, pollen irradiation was used, but alternative methods might be used. For example, segmentation might be induced by other mutagens or be applied at other stages of the life cycle such as interspecific hybrid seed or seedlings. A major consideration for initial experimentation was to minimize chimerism at plant tissue level, which may cause problems during plant tissue collecting and genotyping, for which pollen irradiation holds advantage over seed or seedling irradiation. Chimerism was observed in the RH mapping of maize chromosome 9 where seed irradiation was carried out (RIERA-LIZARAZU et al. 2000). For initial experiments, we used cotton pollen in spite of the fact that it may be inferior to other potential life cycle stages, in terms of the spectrum, plurality and frequency of chromatin deficiencies, due to its relatively higher intolerance to hypo-aneuploidy than somatic and megagametophytic cells.

The fact that cotton is polyploid may have fostered success of this WWRH effort. In spite of its chromosome number, 2n = 52, cotton is disomic (KIMBER 1961), and retains strong genetic redundancy from polyploidy (ENDRIZZI et al, 1984). Genetic redundancy certainly provides relatively greater tolerance to genomic deficiencies and other

imbalances arising from genomic segmentation. Empirically, genomic segmentation will have greater phenotypic impact on genetically reduced haploid cells than on cells at the usual ploidy level. Thus, the relative degree of increased radiation tolerance due to genetic redundancy of polyploidy would expectedly be higher for gametophytes (1n) than for somatic tissues (2n), that is, the ratio of radiation resilience of 2x pollen to 1x pollen might be larger than that of 2n plant to 1n plant. The sensitivity of gametophytes (1n = 2x) and haploid plant (n = 2x) may be unequal at the same ploidy (2x) level. For example, 2x pollen might be more (less) radiation resilient than 2x haploid plants. The sensitivity would likely reflect both the relative number of genes critical to development and survival, capability for DNA repair and the degree of cell autonomy.

We observed significant rates of segmentation and subsequent retention of irradiation fragmented donor chromosomal segments, but at this time we have not determined the physical organization of the retained irradiated segments. At anthesis, when the pollen were irradiated, *Gossypium* pollen are immature, two-celled and binucleate. The second mitosis would have occurred subsequent to pollen irradiation, as it normally occurs in the pollen tube as it grows through the long style (JOSHI et al. 1967; JENSEN and FISHER 1968; FISHER and JENSEN 1969; OOSTERHUIS and JERNSTEDT 1999). McClintock (1941) demonstrated long ago that in maize, microgametophytes and endosperm were subject to breakage-fusion-bridge cycles, whereas zygotes were not. More recent data suggests that this pattern is due to differences in telomerase expression and *de novo* telomere synthesis and that *de novo* telomere synthesis may help stabilize broken chromosomes

(MELEK and SHIPPEN 1996; TSUJIMOTO et al. 1997; SHIPPEN and MCKNIGHT 1998), but it's not known if the same pattern for chromosome "healing" and telomerase expression exists in cotton. Anti-sense constructs, mutants and chemical inhibitors of telomerase might be used advantageously to devise more effective systems for segmentation-based mapping of plant genomes.

Disadvantage of WWRH mapping method: In human and animal species (WG)RH mapping, the efficient chromosome retention frequency was considered to be around 30%. The average marker retention frequency in maize RH mapping was 75-85% (RIERA-LIZARAZU et al. 2000). In this study, we observed retention frequencies from 87% to 94% for individual SSR markers in LG-9. Several possible factors could explain the relatively high retention frequency. Significant selection might occur during the microgametophyte stages, including pollen maturation, pollen germination, pollen tube growth, formation of the germ unit (MOGENSEN 1992), the fertilization process, and so on. Only on rare occasions has cotton pollen been known to transmit a parental deficiency for any whole chromosome or large segment (ENDRIZZI et al. 1985; STELLY, unpublished). Thus, microgametophytic gene transcription and expression is extensive in cotton. Pollen with smaller and fewer deletions are likely to be more viable and more competitive than pollen with larger and more numerous deletions. Stylar splicing (VAN TUYL et al. 1991), placental pollinations (JANSON 1993), or *in vitro* fertilization (reviewed in KRANZ and KUMLEHN 1999) are among the techniques that might be used to raise recovery rates of more deficient pollen, i.e., by alleviating some of the

competitive nature of the fertilization process. *In vitro* embryo rescue could likely be used to increase WWRH recovery from higher irradiation dosages.

In this study, pollen was irradiated at the 2-cell stage, and deletion events would likely have been independent in vegetative and generative nuclei. So, deletion of critical segments from generative (sperm) cells would presumably have not affected pollen viability factors controlled by vegetative nucleus. So, it would seem likely that selection was important during endosperm and embryo development. If cotton is like maize and lacks telomerase in endosperm, the secondary imbalances arising in the endosperm mitoses may often lead to aborted development and thus embryo failure. Of course, failure of embryos due directly to embryo genome deletions or imbalances is certainly also a factor. It's possible that some donor chromosomal segments are acentric and are thus lost during gametophytics or early embryo mitoses. If "unhealed" ends from the donor genome induce interchanges with the recipient genome, not only donor but also exchanged recipient segments might be lost during embryo development, e.g., due to dicentricity or acentricity. Both centromere effects and specific gene effects on marker retention frequency were observed in WGRH mapping method (WALTER et al. 1994).

Radiation dosage is a critically important feature of any irradiation-based segmentation mapping effort. We observed that the 5-krad γ-ray treatment led to more chromosomal breakages, more deletion types and lower chromosomal retention frequencies than 1.5-

krad γ-ray treatment. The dosage effects to be expected in plant WWRH methods need not parallel those in human RH and WGRH methods. Riera-Lizarazu et al. (2000) observed that marker retention frequency decreased nonsignificantly with increasing radiation dosage (RIERA-LIZARAZU et al. 2000), whereas the number of retention patterns increased significantly. No significant marker retention frequency change was observed in chicken with different doses (KWOK et al. 1998). In some cases, higher dosage led to relative higher marker retention frequencies, e.g., in bovine (WOMACK et al. 1997; REXROAD III et al. 2000) and horse (KIGUWA et al. 2000; CHOWDHARY et al. 2002). Our 1.5-krad and 5-krad WWRH data indicated that higher dosages lead to a higher number of retention patterns and lower number of retention frequencies. Our efforts to obtain WWRH plants from 15-krad and 30-krad γ-ray treatment were not successful, so further studies will be required to determine if higher radiation dosages, e.g., 5~15 krads range will improve efficacy by lowering the chromosomal retention frequency and increasing the retention pattern. Given that "multi-hit" events are probably the most common source of interstitial segmental deletions, and those may be the most informative events, the proficiency of WWRH should increase geometrically with dosage. In addition, for this initial effort, we opted to use pollen irradiation as a means of limiting the occurrence of chimerism, other methods such as seedling or meristem irradiation would offer the potential of higher doses and greater fragmentation.

WWRH mapping as a complement to traditional linkage mapping: Traditional linkage mapping is based on the frequency of reciprocal homologous recombination

between markers. The more frequent the recombination between two markers, the greater the linkage map distance between them. However, the estimates of genetic distance between two markers are subject to effects of many factors, such as parental sex, marker density, mapping function, genotypic effects, mapping population, mapping generation, *et cetera*. Like all techniques, linkage mapping suffers from several limitations. One is that linkage maps correlate poorly with physical distances. It is often erroneously assumed that linkage maps provide a guide to the physical layout of chromosomes, i.e., that recombination frequency is more or less evenly distributed along a chromosome. This is important, because most molecular endeavors relate more directly to physical distances (bp) than to linkage maps (cM). However, it has been demonstrated that different chromosome regions may have very different frequencies of recombination, and that the degree of this variation may vary among chromosomes, genomes, and taxa (reviewed in PUCHTA and HOHN 1996; COPENHAVER, et al. 1998; GERTON et al. 2000; reviewed by PETES, 2001; YU et al. 2001). It is well known that recombination around the centromere region is generally suppressed. In cotton, the two-fold disproportionality of A and D subgenomes in terms of DNA content (GEEVER et al. 1989) and chromosome sizes (SKOVSTED 1934; BEASLEY 1942; KIMBER 1961) are not reflected in recombination rates. Existing data show recombination to be very similar in the homeologous pairs (REINISCH et al. 1994). This suggests that A subgenome is recombinationally less active on average than the D subgenome.

The AD genome of cotton is much larger and more complex than that of *Arabidopsis thaliana*, but their average DNA content per cM value are similar (REINISCH et al. 1994; MEINKE et al. 1998, PATERSON and SMITH 1999). In map-based gene cloning in species with small genomes, the problems in chromosome walking that resulted from uneven recombination frequency distribution along chromosomes could be partially overcome by integration of linkage maps with physical maps, in which the distance between markers can be based on actual nucleotide base pair numbers. For example, map-based gene cloning in *Arabidopsis* has become easier now that nearly all of its genome has been sequenced. However, it has been difficult to get physical maps in many chromosome regions good enough to integrate linkage maps in large-genome species. Second, regions that undergo crossing over extremely frequently are difficult to "close" during linkage map synthesis, especially when numbers of polymorphic markers are limited. Conversely, regions that undergo crossing over extremely infrequently form blocks of markers that are recalcitrant to linkage mapping. Also, linkage mapping is like all mapping procedures in that it is prone to errors and misinterpretations. Thus, if used without the benefit of complementary data, linkage mapping will lead to errors and wasteful expenditures of resources. WWRH mapping seems to complement linkage mapping, and to offer some of the generic advantages of other (WG)RH and physical mapping methods.

The results of this research indicate that WWRH mapping could provide a significant complement to traditional linkage mapping in cotton, and perhaps other species. One of

the complementary benefits is that WWRH provides a means to order markers from low-recombination regions. For example, the cotton linkage map (http://demeter.bio.bnl.gov/acecot.html; YU and KOHEL, unpublished data) indicated that microsatellite markers BNL0625 and BNL2805 co-segregated, with no genetic recombination between them in respective linkage mapping populations. In the 5_{krad} WWRH panel, breaks between markers BNL0625 and BNL2805 were identified in two WWRHs, and the order of those two markers along cotton LG-9 was determined. Thus, WWRH mapping offers a facile means of improving our knowledge of locus order beyond that established with linkage maps. In the development of cotton genomic resources, there is a strong need for additional marker loci. For most molecular endeavors, the loci need to be ordered, at least. As larger numbers of markers are developed, increasing numbers of loci will fall into multi-locus bins unresolved by homologous recombination. It's likely that many instances of co-segregation involve markers that are physically distant, but occur in regions where homologous recombination between them is suppressed or reciprocal recombinant products are not recovered -- e.g., peri-centromeric heterochromatin, sub-telomeric repetitive regions, heterochromatic regions replete with highly repetitive sequences, or inversions. By physically disrupting these segments with relatively rare breakage events, WWRH mapping offers a means to order many of the loci in these chromosome regions. Once ordered, these loci can be used more effectively for molecular manipulations, e.g., contig assembly.

A second complementary benefit from WWRH mapping is that it yields maps largely based on physical distances among markers rather than frequency of crossover. Given a radiation dosage, the more base pairs between two markers, the more likely breakage is to occur between them. A radiation hybrid map can be constructed from WWRH marker retention frequency data if it is assumed that breakage and segment transmission are random, evenly distributed and independent. Moreover, radiation hybrid mapping programs offer several models regarding nonrandom transmission or retention of chromosomal segments. For example, the RHMAP program by Boehnke et al (1995) offers four models, equal retention probability model, centromeric retention probability model, left-endpoint retention probability model and general retention probability model. However, it seems likely that even if the breaks occur more or less randomly, that their viability may well differ markedly in many instances, and if so, undermine chances of making reliable estimates of distance. Indeed, loss of key functions governed by loci subject to dosage-sensitivity or extensive parental imprinting would expectedly impart gross effects on recovery of deletions, and thus lead to strong departures from randomness and thus linearity. So, we used the general retention probability model which allows all retention probabilities to differ, even though our data on LG-9 and LG-13 indicated the retention similarity of markers on them. The general retention probability model yielded a WWRH map similar to that from equal retention probability model, but the distances among markers were slightly different (data not shown).

Another benefit of WWRH mapping is that it will foster integration of syntenic linkage groups, assignment of linkage groups to specific cotton chromosomes, and help reduce the number of cotton linkage groups to the gametic chromosomal number (26). In this research, two linkage groups LG-9 and LG-13 in traditional linkage maps were combined into one syntenic group in the WWRH map. Even though this paper did not assign a linkage group to a chromosome independently, WWRH mapping is clearly capable of helping establish chromosomal identity of markers and linkage groups which are syntenic to markers of known chromosomal identity. If marker BNL4053 were not a false positive on LG-11, LG-11 would have been assigned to chromosome 9 by the WWRH mapping method. The integration of LG-9 and LG-13 and the chromosome location assignment of marker BNL4053 by the WWRH mapping method were confirmed by cytogenetic analyses. It is important to notice that complete cytogenetic aneuploid stocks of cotton and many other important crop species are currently unavailable. The WWRH panel itself is a segmentally aneuploid panel and could also serve as a mapping panel complementary to that of conventional aneuploids, as demonstrated already in this research.

WWRH mapping also enables researchers to cross check the accuracy traditional linkage maps. Every method has its own limitations. Results from different methods could be employed to cross-examine each other. This research demonstrated this ability by correcting marker BNL4053 location to chromosome 9 while traditional linkage mapping could located it onto chromosome 1. Most plant species are polyploids with

relatively large and complex genomes. False positives are a common problem in linkage analysis especially at the early stage of map construction when few markers are available. Even at later stage of map development, however, conflicting marker locations or orders are often found among traditional linkage maps. Possible explanations are generally associated with mapping methods, mapping populations and marker types. Orthogonal data derived by methods other than linkage mapping can help resolve such conflictions. For example, our WWRH analyses offered evidences on marker locations along LG-9 where marker location inconsistency existed between traditional linkage maps (http://demeter.bio.bnl.gov/acecot.html; YU and KOHEL, unpublished data).

WWRH clearly offers a facile means of ordering of loci to help map in very low and very high recombination regions of the cotton genome, to coalesce syntenic linkage groups, and to assign markers or linkage groups to chromosomes. Analogous applications seem plausible for other species, particularly those that are functionally polyploid, including those that are relatively recalicitrant to linkage mapping due to genomic complexity, apomixis, polysomy, hybridity, cultivar-specific interests, and/or long generation times.

CHAPTER III

AN 8-KRAD WIDE-CROSS WHOLE-GENOME RADIATION HYBRID (WWRH) MAPPING PANEL OF COTTON (*Gossypium barbadense* L.)

Whole-genome radiation hybrid mapping has been applied extensively to human and certain animal species, but not plants. In lieu of a cell culture system that might enable similar feats in plants, we devised the wide-cross whole-genome radiation hybrid (WWRH) mapping approach. This modified whole-genome radiation hybrid mapping approach was recently demonstrated to be a promising mapping method in cotton (*Gossypium hirsutum* L.), based on segmentation by 5-krad γ-irradiation, but previous results suggested that higher doses might be advantageous. Here, we have sought to develop a second-generation WWRH panel after either 8- or 12-krad irradiation. The genome of *G. hirsutum* (n = 26) was used to rescue the radiation-segmented genome of *G. barbadense* (n = 26) introduced via γ-irradiated pollen. Viable seedlings were not recovered after 12-krad irradiation, so only an 8-krad WWRH mapping panel (N = 92) was constructed. Assessment of 31 SSR marker loci from four chromosomes revealed that the 8-krad panel has a marker retention frequency of *ca.* 76%, which is approximately equivalent to the rate of *loss* in a low-dose animal radiation hybrid panel. Retention frequencies of loci did not depart significantly from independence when compared between the A and D subgenomes, or according to positions along individual chromosomes. WWRH maps of chromosomes 10 and 17 were generated by the

maximum likelihood RHMAP program and the general retention model. The resulting maps bolster evidence that WWRH mapping works in cotton, and that the 8-krad panel complements the 5-krad panel by offering higher rates of chromosome breakages, lower marker retention frequency, and more retention patterns.

Introduction

The cotton genus, *Gossypium*, contains about 50 species. About 45 of those have 2n = 2x = 26 and are widely recognized as diploid. The remaining five species are widely recognized as being of tetraploid origin (2n = 4x = 52). Cytological analyses of chromosome size and meiotic affinity revealed that each 13-chromosome genome could be assigned to a genomic group (A, B, C, D, E, F, G, or K), and that the groups are distributed in a geographically related manner (BEASLEY 1940, 1942; PHILLIPS and STRICKLAND 1966; EDWARDS and MIRZA 1979; ENDRIZZI et al. 1984; PERCIVAL et al. 1999). The A-genome group encompasses two extant African-Asian species and the D-genome group encompasses all extant species in the New World that have 13-chromosome genomes. All five extant 52-chromosome species are of New World origin, and share a more or less similar disomic AD-genome architecture (KIMBER 1961). They are readily hybridized *in vivo* to form relatively fertile F_1 hybrid progenies, though aberrant types occur among subsequent progeny and both segregation and transmission are distorted in subsequent generations (STEPHENS 1949, 1950; BRUBAKER et al. 1999; PERCIVAL et al. 1999). The level of *intra*-specific polymorphism for molecular markers

is relatively low within the two cultivated AD species, *G. hirsutum* and *G. barbadense*, whereas their rates of *inter*-specific polymorphism are much higher. Thus, interspecific hybridization has been used in *Gossypium* to allow for larger numbers of polymorphic loci and thus denser maps (REINISCH et al.1994; YU et al. 1998; ZHANG et al. 2002; MEI et al. 2003).

Genome maps offer extensive advantages for many sorts of genetic endeavors such as integrative genomics, map-based gene cloning, and marker-assisted trait dissection and molecular breeding. Unfortunately, current maps of the cotton genome are relatively immature as compared to some other major crops such as rice (*Oryza sativa* L., 2n = 2x = 24) (GOFF et al. 2002; YU et al. 2002), corn (*Zea maize* L., 2n = 2x = 20) (http://www.maizemap.org/), and wheat (*Triticum aestivum* L., 2n = 6x = 42) (http://wheat.pw.usda.gov/ggpages /maps.shtml#wheat). The number of linkage groups in public linkage maps of the cotton AD genome exceeds its gametic chromosome number (n = 26), and numerous linkage groups have not been assigned to chromosomes (REINISCH et al. 1994; SHAPPLEY et al. 1998; YU et al. 1998 and unpublished data; ULLOA and MEREDITH 2000; ZHANG et al. 2002; MEI et al. 2003). Among the factors that have hindered cotton genome map development include [1] exceptionally high rates of recombination, [2] nonrandomness of crossover distribution, [3] under-utilization of molecular cytogenetic physical mapping, [4] incomplete genome coverage with hypo-aneuploids suited to physical mapping, and [5] reliance on genome-wide marker development strategies.

High rates of recombination in *G. hirsutum* and other *Gossypium* species were first documented by cytological analyses of meiotic pairing in species, interspecific hybrids, and *G. hirsutum* chromosome aberration heterozygotes (SKOVSTED 1934; BEASLEY 1942). These were later augmented by chiasma region analysis (MENZEL et al. 1985), and linkage analysis of molecular markers (REINISCH et al. 1994). Cytogenetic data on *G. hirsutum* meiosis and linkage analysis of interspecific F_1 hybrid indicate that the typical chromosome is almost 200 cM in length, and the AD genome approaches 5000 cM. Many markers are therefore needed to adequately saturate the genome map.

Cytological data have long indicated that the average density of recombination differs between A and D subgenomes of AD species, and that the distribution of reciprocal recombination in 52-chromosome cottons is markedly uneven along the chromosomes. Among 26-chromosome species, chromosomes and DNA content of the A-genome species are more than 1.5-fold larger than are those of the D-genome species (SKOVSTED 1934; BEASELEY 1942; BENNETT AND LEITCH 2003). In spite of the unequal physical sizes of the A and D genomes, the linkage maps of identified homeologues are of similar lengths (REINISCH et al. 1994). Thus, average rates of recombination (cM/Mb) must differ between A and D subgenomes of AD cotton. Nevertheless, the great preponderance of cotton metaphase I chromosome configurations are ring bivalents with chiasmata in distal and/or medial regions of both arms but lacking from regions near centromeres. This is true for chromosomes of A and D subgenomes.

In regions subject to high rates of recombination, the physical density of polymorphic markers must be far higher to robustly detect linkage, unless closure is achieved by an alternative method, e.g., physical mapping. In regions low in recombination, it may be most efficient to use physical mapping approaches to establish linear order. In linkage maps, low recombination regions typically recognized as segments of relatively high marker density and incomplete resolution. Such regions are seen in many higher density maps, including some linkage groups of cotton (REINISCH et al. 1994; ZHANG et al. 2002; MEI et al. 2003). Markers and polymorphism rates tend to be non-random as well, and thus can accentuate or camouflage effects of recombination rate variation on regional marker density within a linkage map. Increased marker density in linkage maps can also result from non-recovery of recombinants and distortions of cryptic cytogenetic aberrations on statistical algorithms built on assumptions of cytogenetic equivalence between mapping parents (LIVINGSTONE et al. 2000). Most public cotton genome maps and mapping resources are relying on the use of interspecific crosses between *G. barbadense* and *G. hirsutum* to achieve high rates of polymorphism. These run a predictable but non-quantifiable risk of inversions, distortions, gametophytic selection and other factors that can compromise the effectiveness of linkage analysis alone for genome mapping. Indeed, marker segregation distortion has been observed in linkage mapping populations of interspecific hybridization (REINISCH et al. 1994; ZHANG et al. 2002; MEI et al. 2003). Even within mapping population of intraspecific hybridization, marker segregation distortion was observed (SHAPPLEY et al. 1998).

Improved resolution and integration of cotton genome maps are very desirable. It is perhaps ironic that the maps can be improved by reduced recombination in certain regions (those with very high rates of crossing over), but increased recombination in others (those with very low rates of crossing over). We previously examined the feasibility of whole-genome radiation hybrid mapping as an approach that could simultaneously address both of these needs. Using radiation-induced chromosome rearrangements to map genes onto chromosome was first demonstrated by Goss and Harris (1975). In contrast to natural recombination, radiation-induced recombination frequencies and map resolution can be modulated by modifying radiation dosages. Reports by Cox et al. (1990) and Walter et al (1994) were key to advancement of radiation hybrid mapping in human and animals. The former (COX et al. 1990) established the "radiation hybrid (RH) mapping method", in which only one chromosome is mapped per radiation hybrid mapping panel. The second (WALTER et al. 1994) improved efficiency by establishing the "whole-genome radiation hybrid (WGRH) mapping method", in which all chromosomes are mapped per radiation hybrid mapping panel. Radiation hybrid mapping not only increased the recombination events, but it offered the advantages of very high rates of polymorphism between donor and recipient cell lines. With radiation hybrid mapping, it became possible to use markers monomorphic within the donor species for mapping (COX et al. 1990). The (WG)RH mapping approach has been rapidly assimilated into genome mapping efforts for humans and certain animal species (MCCARTHY 1996; SCHMITT et al. 1996; MCCARTHY et al.

1997; WOMACK et al. 1997; KWOK et al. 1998, 1999; PRIAT et al. 1998; YERLE et al. 1998; GEISLER et al. 1999; HAWKEN et al. 1999; MURPHY et al. 1999; VIGNAUX et al. 1999; WATANABE et al. 1999; KIGUWA et al. 2000; REXROAD et al. 2000; OLIVIER et al. 2001).

All forms of radiation hybrid mapping method have had extremely limited application in plant species. Perhaps the chief obstacle to widespread application in plant species is the difficulty of finding appropriate recipient cell lines to rescue irradiation-fragmented plant chromosomes. In lieu of such cell lines, whole plants have been used. RIERA-LIZARAZU et al. (2000) reported an RH mapping approach for maize, which uses oat maize-chromosome addition lines to map maize chromosomes. The maize approach is applied to individual maize chromosomes and is thus roughly equivalent to the single-chromosome approach reported by Cox et al. (1990). To achieve greater efficiency and wider applicability, we developed a whole-genome approach analogous to the WGRH methods of Walter et al. (1994), using cotton as the target organism. To distinguish the method from other radiation hybrid mapping methods, we dubbed it wide-cross whole-genome radiation hybrid (WWRH) mapping. In WWRH mapping, a wide-cross is used to rescue irradiation-fragmented genomes and to elevate polymorphism levels. For initial characterization, pollen irradiation was used to fragment just one genome of the hybrids in the WWRH panel. Although we recognized that irradiation at additional or other stages, e.g., megagametophytic, and/or zygotic, might offer higher resolution, pollen irradiation was simple and expected to minimize chimerism.

Similar to the animal WGRH method, the plant WWRH method should enable mapping of the whole genome with one mapping panel. In contrast to the WGRH method, where *in vitro* radiation hybrid cell lines comprise the radiation hybrid mapping panel, a WWRH mapping panel is created *in vivo* through wide crossing. In the seminal WWRH development, the genome of *G. barbadense* (n = 26) was used to rescue radiation-segmented genome of *G. hirsutum* (n = 26) introduced via γ-irradiated (5-krad) pollen. Analysis of the WWRH mapping panel revealed that: (1) WWRH works in cotton, and that (2) WWRH complements traditional linkage mapping, especially in genome regions with relatively high or low recombination frequency. The initial findings also indicated that the WWRH gave observed marker retention frequencies that were approximately equivalent to the rate of *loss* in low-dose animal (WG)RH panels, suggesting that statistical efficacy might be similar. Initial results also indicated that a radiation dose between 5 krads and 15 krads could optimize the retention frequencies for WWRHs for 52-chromosome cottons.

In this chapter, we present results of a dosage study and the characteristics of a newly constructed 8-krad WWRH panel. Analysis of the 8-krad panel further demonstrates that WWRH mapping works in cotton and complements linkage mapping, and that multiple WWRH panels complement each other.

Materials and methods

Plant materials: Two cotton lines, TM-1, a highly inbred line of *Gossypium hirsutum* L. (2n = 4x = 52) with two $(AD)_1$ genomes, and 3-79, a doubled haploid line of *Gossypium barbadense* L. (2n = 4x = 52) with two $(AD)_2$ genomes, were chosen as parents to generate a new WWRH panel(s). TM-1 served as the female parent, and 3-79 served as the male parent. They were selected as parents because they are highly homozygous, have been used extensively as parents for linkage mapping (YU et al. 1998), recombinant inbred line development, large-insert genomic library development and introgression via chromosome substitution. Relatively high molecular marker polymorphism exists between TM-1 and 3-79, and both are related to currently cultivated Pima and Upland cotton cultivars.

Radiation treatments: Flowers of 3-79 at anthesis were irradiated with 8 or 12 krads of γ-rays at the Texas A&M University Nuclear Science Center, then pollinated to TM-1 flowers emasculated the day before. For each dosage, about 400 cross-pollinations were made. The number of F_1 seed, F_1 seed germination percentage, number of viable F_1 seedlings or plants, and F_1 plant chromosome deletion types and deletion frequencies were surveyed as described below.

Mapping panel construction and genotyping: A mapping panel was constructed from hybrids formed after interspecific cross-pollination of non-irradiated *G. hirsutum*

emasculated flowers by 8-krad irradiated *G. barbadense* pollen. To characterize the 8-krad mapping panel, the panel was screened with 31 polymorphic SSR markers from chromosomes 10, 12, 17, and 18 that have been used to create a linkage map (YU and KOHEL, unpublished). Chromosomes 10 and 12 are from the A subgenome and chromosomes 17 and 18 are from the D subgenome. Previous WWRH mapping results (5-krad panel) indicated that chromosome 17 includes at least three linkage groups of the traditional linkage map by Yu and Kohel (unpublished), i.e., Chr. 17, linkage group 9 (LG-9) and linkage group 13 (LG-13). The association of all three linkage groups to chromosome 17 was confirmed previously by deficiency tests with monosomic interspecific hybrids lacking chromosome 17 (Chapter II).

DNA samples were extracted from young leaf tissues of two-month old plants. SSR primers were purchased from Research Genetics Inc. Each PCR reaction included 100 ng genomic DNA, 0.2 mM dNTPs, 3.0 mM $MgCl_2$, 0.04 μM forward primer, 0.04 μM reverse primer and 0.5 unit of Taq polymerase in 15 μl total reaction volume. PCR reactions were performed as follows: 95 °C for 2 min; 40 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min; 72 °C for 7 min; and 4 °C for holding. PCR products were resolved in 4% agarose gels. WWRHs missing 3-79 PCR amplification products were identified as deletion lines (Figure 7). Experimental controls included the two parents, 3-79 and TM-1, as well as a non-irradiated TM-1 x 3-79 F_1 hybrid.

Figure 7: Principle of WWRH genotyping. Marker A is a co-dominant marker between parents TM-1 (female parent) and 3-79 (source of irradiated pollen). Normal F_1 hybrid is heterozygous for both parental alleles. WWRH1 is a deletion line for marker A because the marker A band from parent 3-79 is missing, while WWRH2 is not a deletion line for marker A because the marker A band from parent 3-79 is present. All WWRHs should contain markers from the homozygous female parent TM-1, e.g., the upper (TM-1) marker A band. The maternal bands should be present in all WWRHs so they serve as an experimental control for normal PCR reaction, e.g., in WWRH1.

Statistical analysis: The presence/absence (+/-) of each marker was scored for each WWRH, then marker retention frequencies were calculated in the respective WWRH panel. The retention frequency for a given marker was calculated simply as the ratio between the number of WWRH plants carrying the marker band to the total number of WWRH plants in that panel that were unambiguously genotyped. Chi-square tests on all 31 SSR markers from four chromosomes were carried out to determine if SSR marker retention frequency was independent of their locations. A t-test was carried out to see whether there were significant differences between the A and D subgenomes for marker retention frequency. Radiation hybrid mapping program RHMAP version 2.01 (BOEHNKE et al. 1995) was also used to analyze the genotypic data. The two-point RHMAP analyses were carried out on all 31 SSR markers to identify syntenic groups. The minimum LOD-score of 4.0 was used as the criterion to claim significance. The syntenic groups generated from RHMAP analyses were compared and contrasted with those from the traditional linkage maps (YU et al. 1998; YU and KOHEL unpublished). The maximum likelihood RHMAP analysis with the general retention model was carried out to generate WWRH maps on certain identified syntenic groups from the two-point RHMAP analyses.

Results

Dosage effect analysis: Similar quantities of seed were formed after cross-pollination with γ-irradiated pollen for 8- and 12-krad dosages. The seed were seemingly normal in

appearance in that they had black or brown-black seed coats and were approximately normal in size. However, the quality of seed differed markedly between the two radiation dosages. From the 1506 8-krad seed, 144 germinated and yielded 92 viable seedlings. Phenotypic variation among the 8-krad WWRHs was marked (Figure 8). The rate of seed germination was unusually variable. Most seed (96) germinated after incubation at 37 °C for two days, whereas 24, 17, and seven seed germinated after three, four, and five days, respectively. From the ~1200 12-krad WWRH seed, most seed were found to be hollow, and none germinated.

Genotyping: All 92 WWRHs from the 8-krad treatment were used to construct the 8-krad WWRH mapping panel. The panel was genotyped with 31 SSR markers, of which six, four, 15, and six markers were from chromosomes 10, 12, 17, and 18, respectively. The genotypes were listed in Table 5. Chromosomes 10 and 12 are from the A subgenome and chromosomes 17 and 18 are from the D subgenome. Chromosome 17 includes three linkage groups, of which only one (Chr. 17) was assigned to chromosome 17 in traditional genetic linkage map while two linkage groups (LG-9 and LG-13) were assigned to chromosome 17 according to our pilot investigation of WWRH mapping using the 5_{krad} mapping panel (Chapter II).

Figure 8: Picture showing significant phenotypic variation among WWRHs. Picture was taken about 45 days after seed germination.

TABLE 5

WWRH genotypes

WWRH ID / BNL	2960	1161	3563	3895	1665	0256	1679	1673	2967	3261	3955	2443	1681	3411	1404	3431	1034	3442	4094	2632	2805	1408	3592	1066	0836	2652	3280	3479	1079	3558	2544
Chr	10	10	10	10	10	10	12	12	12	12	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	18	18	18	18	18	18
RF(%)	83.70	91.30	86.96	83.70	90.22	78.26	77.17	76.09	90.22	80.43	95.65	96.74	75.00	70.65	70.65	69.57	68.48	65.22	70.65	71.74	72.83	85.87	85.87	80.43	82.61	94.57	96.74	95.65	98.91	95.65	93.48
902705	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
902706	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
902707	1	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
902708	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
902709	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
902710	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1
902711	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902712	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
902713	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902714	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
902715	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
902716	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902717	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
902718	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
902719	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902720	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
902801	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
902802	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902803	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902804	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902805	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
902806	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
902807	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902808	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902809	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902810	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
902811	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902812	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902813	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1
902814	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
902815	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

TABLE 5 (continued)

BNL	2960	1161	3563	3895	1665	0256	1679	1673	2967	3261	3955	2443	1681	3411	1404	3431	1034	3442	4094	2632	2805	1408	3592	1066	0836	2652	3280	3479	1079	3558	2544
WWRH ID / Chr	10	10	10	10	10	10	12	12	12	12	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	18	18	18	18	18	18
902816	1	1	1	0	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
902817	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
902818	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1
902819	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1
902820	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902901	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1
902902	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
902903	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
902904	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1
902905	1	1	1	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902906	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902907	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902908	0	0	0	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
902909	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902910	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
902911	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902912	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
902913	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1
902914	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902915	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
902916	0	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
902917	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1
902918	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
902919	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1
902920	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903001	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
903002	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
903003	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
903004	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903005	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
903006	0	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
903007	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

TABLE 5 (continued)

WWRH ID / BNL	2960	1161	3563	3895	1665	0256	1679	1673	2967	3261	3955	2443	1681	3411	1404	3431	1034	3442	4094	2632	2805	1408	3592	1066	0836	2652	3280	3479	1079	3558	2544
Chr	10	10	10	10	10	10	12	12	12	12	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	18	18	18	18	18	18
903008	1	1	1	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
903009	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
903010	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903011	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903012	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903013	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903014	0	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
903015	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
903016	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903017	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
903018	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1	1
903019	0	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
903020	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1
903101	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	1	1	1	1
903102	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
903103	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1
903104	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1
903105	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903106	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903107	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903108	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
903109	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
903110	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
903111	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
903112	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903113	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903114	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903115	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
903116	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

0 = deleted, 1 = undeleted.

Using the 31 SSR markers, 76 of the 92 WWRH plants were identified as deletion lines (Table 5), of which, 11, 9, 10, 4, 4, 6, 1, 4, 4, 6, 3, 8, 4, 1, and 1 deleted one, two, three, four, five, six, seven, eight, nine, ten, twelve, thirteen, fourteen, fifteen and sixteen marker(s). For individual WWRH, the maximum number of markers deleted was 16. For individual SSR marker loci, the retention frequencies ranged from 65.2 to 98.9% with an average of 76.1%. Chi-square test indicated that marker retention frequency was independent of marker locations ($\chi^2 = 0.6107 < \chi^2_{\alpha = 0.05, v = 30} = 43.77$). For the A subgenome, the average marker retention frequency was 83.8% with a range of 76.1% ~ 91.3% according to 10 SSR markers, and for the D subgenome, 82.7% and 65.2% ~ 98.9% according to 21 SSR markers. A t-test indicated that no significant difference existed between A and D subgenomes for marker retention frequencies ($t = 0.2738 < t_{\alpha = 0.05, v = 29} = 2.045$).

RHMAP analysis: The syntenic groups identified at LOD score 4 are listed in Table 6. Twenty-four out of 31 SSR markers were found syntenic to one or more SSR markers at LOD-score 4. One, one, two and two syntenic group(s) were identified for chromosome 10, 12, 17, and 18, respectively. No markers from different chromosomes were found syntenic during RHMAP analyses.

WWRH maps of syntenic groups with more than three markers were generated using the maximum likelihood RHMAP analysis with the general retention model, one each for chromosomes 10 and 17 (Figure 9). The WWRH map lengths for syntenic groups from

TABLE 6

Linkage groups identified at LOD-score 4

Linkage groups (Chromosomes)
BNL2960 BNL1161 BNL3563 BNL3895 BNL1665 (Chr 10)
BNL1673 BNL1679 (Chr 12)
BNL4094 BNL2632 BNL2805 BNL1681 BNL3411 BNL1404 BNL3431 BNL1034 BNL3442 (Chr 17)
BNL1408 BNL3592 BNL1066 BNL836 (Chr 17)
BNL 2544 BNL3558 (Chr 18)
BNL3280 BNL3479 (Chr 18)

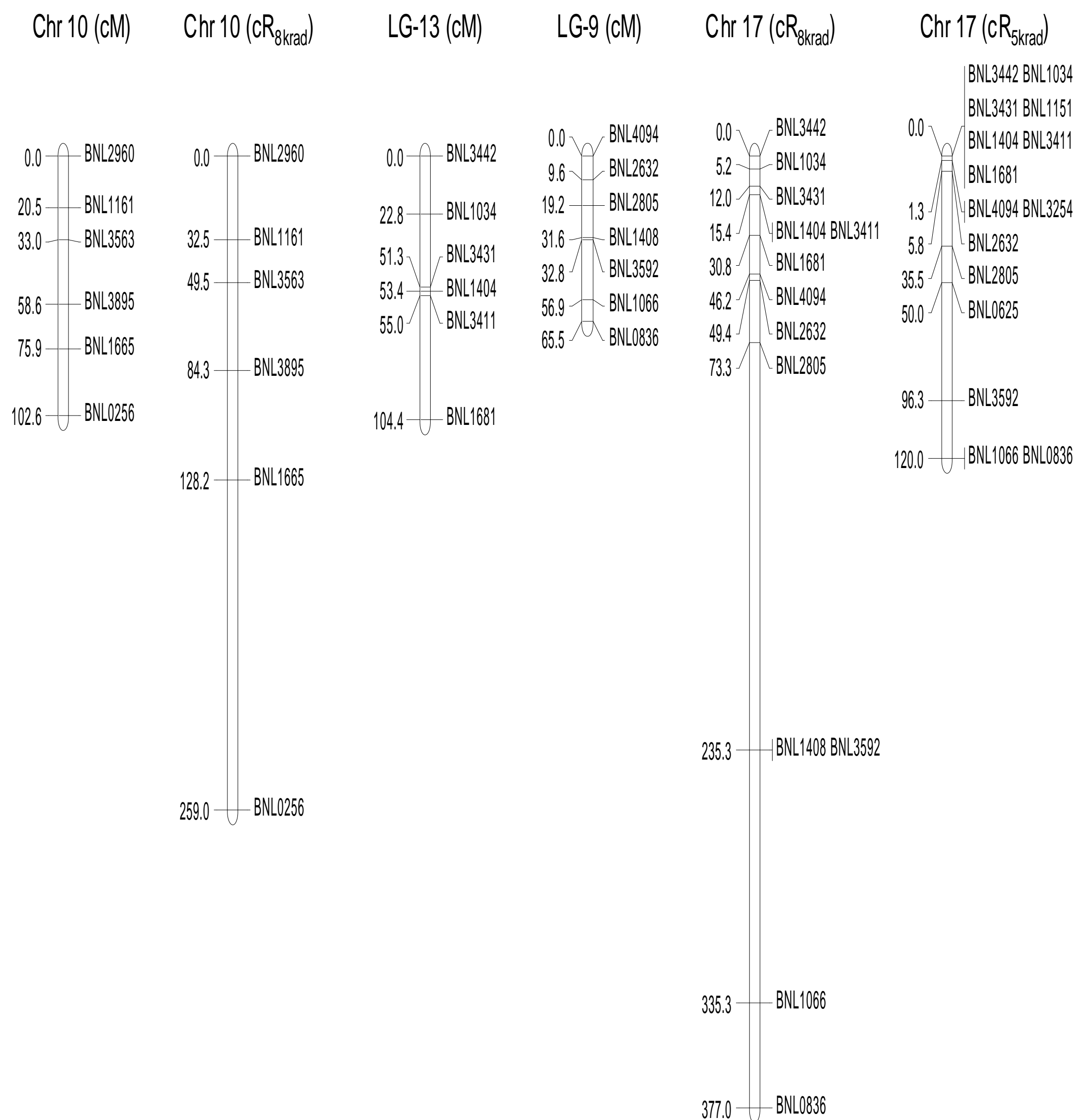

Figure 9: Map comparison among a traditional linkage map (YU and KOHEL, unpublished), the 8_{krad} WWRH map, and the 5_{krad} WWRH map. Two linkage groups (LG-9 and LG-13) were mapped into one linkage group using both the 5_{krad} and the 8_{krad} WWRH mapping panels.

chromosomes 10 and 17 were 250.9 and 377.0 centiRays$_{8krad}$, respectively. The marker distance correlation coefficient between the traditional linkage map (YU, unpublished data) and the WWRH map was 0.7054, 0.6567 and 0.9597 for chromosome 10, LG-9 and LG-13, respectively.

Discussion

Complementation of 8$_{krad}$ and 5$_{krad}$ WWRH panels: In contrast to the 5$_{krad}$ mapping panel, the 8$_{krad}$ mapping panel offers several advantages, including more breakages and more deletion patterns. For the 5$_{krad}$ panel, the retention frequencies are similar for markers from LG-9 and LG-13. However, in the 5$_{krad}$ WWRH panel, we observed more disparity in map resolution among chromosomes. Whereas most LG-9 markers were resolved, LG-13 markers were not resolved in the 5$_{krad}$ panel. In the 8$_{krad}$ panel, markers of LG-13 were well separated and map resolution was relatively uniform among chromosomes. Most deletion lines from the 5$_{krad}$ panel missed one or a few markers, often from a single chromosome or linkage group. Based on 31 SSR markers from four chromosomes, 82.6% WWRHs (76 out of 92) of the 8$_{krad}$ panel were deletion lines, 67 unique hybrid retention patterns were observed among 92 WWRHs, and most deletion lines (43 out of 76) missed markers from two or more chromosomes (Table 5). We surmise that low dosage largely results in chromosome or large chromosomal segment deletions, whereas higher dosage causes a lot of chromosomal breakages and

recombination events, and leads to more patterns of retention or deletion. We compared deletion patterns across both panels using common LG-9 markers. The 5_{krad} panel offered 10 different deletion types out of 101 WWRHs, whereas the 8_{krad} panel gave at least 15 deletion types out of 92 WWRHs (Table 5). The 8_{krad} panel contained all deletion patterns found in the 5_{krad} panel, but not vice verse. So, we believe the 8_{krad} panel offers higher map resolution than the 5_{krad} panel. Because numbers of linkage groups in current cotton maps exceed the gametic chromosome number, and about half the linkage groups are yet to be associated to specific chromosomes. The 5_{krad} panel will be especially helpful for linkage group coalescence, theoretically better than the 8_{krad} panel, in which there are more breaks per unit length of chromosome. For example, the map length of chromosome 17 from marker BNL3442 to BNL0836 was increased from 120 $centiRay_{5krad}$ to 377.0 $centiRay_{8krad}$. While the 8_{krad} panel offered higher resolution, it also exhibited far more phenotypic variance and required more effort to establish and maintain.

Radiation dosage: Radiation dosage is a critically important feature of any irradiation-based segmentation mapping effort. We observed the 8-krad γ-ray treatment led to more chromosomal breakages, more deletion types and lower chromosomal retention frequencies than the 5-krad γ-ray treatment. The dosage effects to be expected in plant WWRH methods need not parallel those in human RH and WGRH methods. Riera-Lizarazu et al. (2000) observed in maize RH panels that marker retention frequency decreased nonsignificantly with the increasing of radiation dosage, whereas the number

of retention patterns increased significantly. No significant change in marker retention frequency occurred in chicken WGRHs developed with different doses (KWOK et al. 1998). In some cases, higher dosage has led to relative higher marker retention frequencies in bovine (WOMACK et al. 1997; REXROAD III et al. 2000) and horse (KIGUWA et al. 2000; CHOWDHARY et al. 2002). In cotton, a significant increase in marker retention **patterns** was observed among WWRHs from increased radiation dosage. Although increased radiation dosage led to a decreased marker retention frequency among WWRHs, the 8_{krad} panel marker retention frequency is roughly equivalent to that from only a low-dose irradiated animal (WG)RH mapping panel. It's likely that the *in vivo* rescue process in cotton WWRHs generally requires higher gene retention frequency than animal *in vitro* (WG)RHs because the WWRHs must be both cell-viable and more or less developmentally capable. The genetic constraints would impose certain limitations on chromosome deletions. This might also be true in maize RHs, for which the seed must be able to germinate and grow. We believe that the cotton WWRH marker retention frequency is sufficiently high for mapping, yet the genome might be further dissected with higher cumulative radiation doses. We propose that the marker retention frequency is less important than marker retention pattern, and that increasing the number of marker retention patterns through adjusting radiation dosage could further increase the WWRH mapping power for high-resolution mapping purposes. For example, a cumulative pollen irradiation dosage between 8 krads and 12 krads might be used very advantageously to further increase the marker retention patterns in cotton, even though the marker retention frequency might not shift

significantly toward the levels as observed in human and some animal species. Most current cotton maps have relatively low resolutions, so both the 5_{krad} and 8_{krad} panels can be used to increase map resolution and help integrate mapping resources. Development of a higher dosage panel, e.g., 10-krad, would be more difficult and time-consuming, but it would likely exhibit even more variation in retention patterns and thus higher map resolution.

Application of the WWRH mapping as a complement of traditional linkage mapping: Like (WG)RH mapping methods, the WWRH mapping approach complements traditional linkage mapping by offering the following advantages: [1] It enables ordering of markers from segments that are exceptionally low or high in recombination. Traditional linkage mapping is based on the frequency of reciprocal homologous recombination between markers. One significant limitation of linkage maps is that they correlate poorly with physical distances. Ironically, it is often assumed that linkage maps provide a guide to the physical layout of chromosomes, i.e., that recombination frequency is more or less evenly distributed along a chromosome. It has been demonstrated that different chromosome regions may have vastly different frequencies of recombination, and that the degree of this variation may vary among chromosomes, genomes, and taxa (reviewed in PUCHTA and HOHN, 1996; COPENHAVER, et al. 1998; GERTON et al. 2000; reviewed by PETES, 2001; YU et al. 2001). In AD-genome cotton species (2n = 52), the DNA content (GEEVER et al. 1989) and chromosome sizes (SKOVSTED 1934; BEASLEY 1942; KIMBER 1961) of the A subgenome

are larger than those of the D subgenome, whereas mapping data show almost identical recombination lengths of A and D subgenomes. This discordance suggests that the A subgenome overall undergoes less recombination per DNA unit than the D subgenome (REINISCH et al. 1994). Regions that undergo crossing over extremely infrequently form blocks of markers that are recalcitrant to linkage mapping. Conversely, regions that undergo crossing over extremely frequently are difficult to "close" during linkage map synthesis and can result in unexpectedly large numbers of linkage groups, some of which are syntenic. Under assumptions of randomness and independence of chromosomal breakage and segment transmission, the cotton WWRH maps were generated largely based on physical distances among markers rather than biological recombination frequencies. The 5_{krad} and the 8_{krad} WWRH maps demonstrate that the WWRH mapping method is able to complement linkage mapping by ordering markers from low recombination frequency regions, and coalescing syntenic linkage groups that flank high recombination frequency regions. [2] It permits the manipulation of map resolution by control of radiation dosage. Whereas it is difficult to manipulate the biological recombination frequency during genetic linkage mapping, it is relatively easy to manipulate chromosome segmentation by modifying radiation dosage in the WWRH mapping. With increased radiation dosage, more breaks and thus longer map distances are expected between two given markers. For example, the marker distances calculated from the 8_{krad} panel are generally much larger than those from the 5_{krad} panel (Figure 9). Similarly, map distances can be reduced by decreasing the radiation dosage. Higher dosages can be used to order co-localized markers in linkage map, and lower dosages

can be used to integrate linkage groups into chromosomes. Both applications are relevant to contemporary cotton genome mapping efforts. Most cotton genome maps feature regions rather disparate in marker density, and include more linkage groups than the AD cotton genomes have chromosomes. [3] WWRH mapping is applicable to wider crosses than linkage mapping, and thus theoretically capable of mapping a relatively larger number of loci, since rates of polymorphism will be higher. WWRH mapping requires the recovery of viable WWRH hybrids from wide crosses to generate mapping populations, but the hybrids need not be fertile. In contrast, traditional linkage mapping requires not only the formation of F_1 hybrids, but also that they be pollen- and/or ovule-fertile. [4] WWRH can be applied without regard to cytogenetic differences between the parents, whereas cytogenetic differences are expected to distort linkage-mapping populations and thus create artifacts within conventionally computed linkage maps. We anticipate that WWRH will enable the detection and localization of structural rearrangements between linkage mapping parents, and when suitably interfaced with linkage mapping software, will provide a collective product that is more robust than possible by linkage mapping alone. [5] WWRH can be used to generate a genome map relatively quickly. For plant species, the most popular linkage mapping populations are F_2 populations, backcrossing populations, and recombination inbred lines. In the WWRH method, the quasi-F_1 plants are well suited for mapping without additional generations. This advantage could be especially significant for plant species with long life cycles.

Several limitations are certain to affect usage of WWRH mapping. One of these is radiation sensitivity. Some genomes, genotypes, and specific chromosomal regions might be more or less fragile to radiation treatment than others. At a given stage of the life cycle, the genetic losses induced by radiation will be less debilitating at higher ploidy levels, and most debilitating at diploid and monoploid ones. Polyploid plants will expectedly prove more amenable to WWRH mapping than diploid ones. Existing data on fertility and transmission of hypo-aneuploidy in cotton and other species suggest that differential effects of gene loss are more extreme at the microgametophytic stage than at megagametophytic or sporophytic stages. For example, monosomy and monotelodisomy in cotton are hemizygous states that are much more readily transmitted via mega- than micro-gametophytes. Pollen-mediated transmission is zero or near zero for most cotton hypo-aneuploids. The amenability of pollen to irradiation and the WWRH approach thus seem somewhat surprising. A significant factor favoring success of our work, however, may be that irradiation was applied near anthesis, i.e., after the first mitotic division, so deficiencies in the generative nucleus would have arisen independently of those which might affect the vegetative nucleus and most pollen functions. While pollen irradiation works well for the AD 26-chromosome genomes of *G. hirsutum* and *G. barbadense*, other methods might be more appropriate in some other species. Irradiation or other means of inducing chromosome breakage might be applied more advantageously at other points in the life cycle. For species in which radiation sensitivity is too high, such as might be true for obligately allogamous species with high genetic load, the amount or inducible recombination may be insufficient to warrant use of WWRH. [2] Strong

regional differences in the genome to the deficiencies induced by irradiation could lead to highly nonrandom transmission of broken chromosomal segments. Both centromere effects and specific gene effects on marker retention frequency were observed in the WGRH mapping method (WALTER et al. 1994). We believe the centromere effects and the loss of key function genes for proper seed and seedling development are also important limitations in our WWRH method. Both limitations could lead to map departures from linearity in distance estimates. Although our data suggested marker retention similarity on both the 5_{krad} and the 8_{krad} mapping panels, we generated the WWRH maps by using the general retention probability model, which allows all retention probabilities to differ.

There has been extensive use of intergeneric and interspecific hybridization among various plant taxa to increase genetic diversity, to construct genetic analysis stocks, and even to generate new species such as triticale. WWRH mapping takes advantage of the feasibility of interspecific hybridization among many related plant species. As such, it offers a physical approach to mapping that is quick, simple and inexpensive and effective. It maps the whole genome with a single WWRH mapping panel. The *in vivo* rescue process of wide crossing is simpler than the *in vitro* rescue process that has been used to generate mapping panels of human and animal genomes. It is applicable to the genome of cotton and perhaps many other plants. Analogous WWRH applications seem plausible for other species, particularly those that are functionally polyploid, including

those that are relatively recalicitrant to linkage mapping due to genomic complexity, apomixis, polysomy, hybridity, cultivar-specific interests, and/or long generation times.

CHAPTER IV

IDENTIFICATION OF COTTON (*Gossypium hirsutum* L.) GENES INDUCED BY COLD TEMPERATURE USING 70-MER OLIGO-GENE MICROARRAY ANALYSIS

Plant growth and development are affected by various environmental stresses. Cold stress is a common abiotic environmental stress in plants. Cold tolerance in cotton is a complex trait controlled by many genes. Little is known about the mechanisms of cold-induced gene expression in cotton. However, a large number of stress-related genes have been identified and characterized in the model plant *Arabidopsis*. In this study, we employed spotted oligo-gene microarrays to identify cold-responsive genes in germinating cotton seed. The 1,536 70-mer oligos were designed from cotton cDNAs and ESTs, of which 806 showed homology to drought/cold induced orthologous genes in *Arabidopsis* and other plants. Using the cotton oligo-gene microarray, we identified 38 cold-responsive cDNAs or ESTs, of which 36 were up-regulated 1.37- to 2.92-fold, and two were down-regulated 1.35- and 1.89-fold. The candidate genes encode putative proteins homologous to late embryogenesis abundant proteins, water channel proteins, cell division related proteins, chloroplast related proteins, and heat shock proteins. Northern blot analyses confirmed the expression level of 16 candidate genes. Our data suggest that *Arabidopsis* orthologous genes can be used for identification of candidate genes in cotton and that 70-mer oligos are attractive alternative to cDNA amplicons for analysis of gene expression by means of microarray analysis.

Introduction

Plants grow in highly variable environments and are subject to various environmental stresses throughout their life cycles including biotic and abiotic stresses. The most significant abiotic environmental stresses on plant species worldwide are drought, cold and salt. Plants respond to their environmental stresses at various levels such as morphological and physiological changes, and the responses also depend on stress duration and severity, plant genotypes, tissue types, and developmental stages. Different crops may have different responses to environmental stress. For example, the developmental growth and yield of many species can be reduced severely by even moderate water stress (HEATHERLY et al. 1977).

Changes in gene expression in response to stress have been studied in many crop species such as maize (ZINSELMEIER et al. 2002), wheat (SIVAMANI et al. 2000), rice (SALEKDEH et al. 2002), tomato (KAHN et al. 1993), tobacco (FERRARIO-MERY et al. 1998), and cotton (ROARK et al. 1973; FENDER and O'CONNELL 1989; GALAU et al. 1992; PENNA et al. 1998). A given stress condition will alter expression of a group of genes. Some genes can be induced by different stresses. This suggests that gene interactions and common pathways exist among the stress response systems. For example, some drought-induced genes are expressed (1) under cold and salt; (2) after ABA treatment; and (3) during late stages of embryogenesis (BAKER et al. 1988; KAHN et al. 1993; YAMAGUCHI-SHINOZAKI and SHINOZAKI 1994; MANTYLA et al. 1995; XU et al. 1996; BRAY 1997; SHINOZAKI and

YAMAGUCHI-SHINOZAKI 2000; TAJI et al. 2002). Stress-induced genes can be divided into two functional groups: (1) those directly protecting plants against environment stresses, and (2) those involved with regulation and signal transduction of environmental stress responses (BRAY 1997; SHINOZAKI and YAMAGUCHI-SHINOZAKI 1997; THOMASHOW 1999; HASEGAWA et al. 2000). Tolerance to environmental stresses is a complex multigenic trait (SAKAI and LARCHER 1987; FOWLER and THOMASHOW 2002). Thus, understanding the plant stress tolerance process requires a comprehensive evaluation of expression for a large number of stress-inducible genes. Strategies to identify stress inducible genes typically rely on differential screening of mRNAs, proteins, and/or cDNA libraries from both stressed and non-stressed materials. The most frequently used screening materials are cDNA libraries, but the screening process they entail is labor-intensive and time-consuming. More efficient and high-output approaches are desirable.

Development of microarray technology (SCHENA et al. 1995; LOCKHART et al. 1996) has made it feasible and convenient to screen gene expression patterns on a genome-wide scale. Microarray technology is starting to become widely used in functional genomic studies of plants, especially for the analyses on differentially expressed genes. Oligonucleotide or cDNA arrays on one slide can be hybridized simultaneously to two different fluorochrome-labeled probes generated from different treatments such as different cell types and different tissues. The comparative nature of two-color microarray analysis is well suited to the study on differential gene expression. Microarray technology has been

used to identify many genes induced by various environmental stress conditions (SEKI et al. 2001; KREPS et al. 2002; OONO et al. 2003). Using fluorochrome-labeled probes prepared from stress-treated cotton tissues and non-stressed controls, microarray analysis should allow us to detect stress-related gene expression patterns between two treatments.

Model organisms have been adopted widely for diverse biological studies. The relative availability of resources for large-scale gene-expression analysis in model organisms such as microarrays (SCHENA et al. 1995; LOCKHART et al. 1996) and serial analysis of gene expression (VELCULESCU et al. 1995) are fueling this trend (SEKI et al. 2001; KREPS et al. 2002; OONO et al. 2003). Relative to the genomic simplicity of the model plant species *Arabidopsis*, genomic complexity of other species makes it all the more important that techniques for large-scale gene expression analysis be adapted to them. This need is increasing the research emphasis on how to benefit genomic studies on non-model species from genomic studies on *Arabidopsis*. Various types of microarrays have been developed for *Arabidopsis*, while the availability of microarrays for other plant species has been very limited. In lieu of species- or genus-specific microarrays, some researchers have used *Arabidopsis* microarrays to study other plant species (HORVATH et al. 2003). While such applications offer some comparative and within-species insight, they do not obviate the need for species- or genus-specific microarrays.

Cold stress is among the most important abiotic stresses in cotton production. Cold stress in early developmental stage often renders cotton susceptible to fungal infection and

many other environmental stresses. Cold tolerance in cotton would expand the cotton growing season, increase tolerance to other stresses such as seedling diseases, and increase fiber yield and quality. In contrast to hundreds of genes or ESTs identified in *Arabidopsis*, only a few genes have been reported as drought- and/or cold-inducible in cotton. Most cotton genes were identified by traditional genetic methods from drought-stress experiments. None of them has been characterized in detail at the molecular level. Identifying and characterizing cotton genes related to cold-tolerance will facilitate development of cold-tolerant cotton cultivars, either through marker-assisted breeding and(or) transgenic approaches. Increased stress-tolerance levels can be achieved by genetic engineering through transgenic approaches (JAGLO-OTTOSEN et al. 1998; KASUGA et al. 1999; THOMASHOW 1999).

Our approach to the identification of cold-response genes in cotton is based on the fact that genes with similar functions often have conserved domains among different species. This was vividly illustrated by Horvath et al (2003), who recently demonstrated that many differentially expressed genes from distantly related taxa were conserved and could be readily monitored directly using *Arabidopsis* microarrays. We chose an alternative strategy that also makes use of *Arabidopsis* data. We developed a cotton oligo-gene microarray based on sequence homology between cotton and *Arabidopsis* genes. Because genes induced by cold stress are often induced by drought stress (SEKI et al. 2001; KREPS et al. 2002), drought- and/or cold-inducible genes of *Arabidopsis* were used as reference genes to identify homologous cotton cDNAs and ESTs, from which

70-mer oligos were designed to develop cotton oligo-gene microarrays. Here, I present our research on identification of cotton genes responsive to cold stress using the cotton 70-mer oligo-gene microarray. The results clearly demonstrate the feasibility of incorporating functional genomic information from *Arabidopsis* into studies on other plant species.

Materials and methods

Cotton 70-mer oligo design: Ninety-three drought/cold inducible genes were selected based on previously published data (Table 7). The gene list includes 62 genes from *Arabidopsis thaliana*, three from *Brassica napus*, three from *Craterostigma plantagineum*, 10 from *Gossypium*, one from *Glycine max*, four from *Hordeum vulgare*, one from *Lycopersicon chilense*, two from *Lycopersicon esculentum*, one from *Mesembryanthemum crystallinum*, one from *Medicago sativa*, two from *Pisum sativum*, and three from *Triticum aestivum*. Using the translated sequences, we searched for orthologous genes in cotton databases from GenBank that contains about 16,000 ESTs and cDNAs and from the Institute for Genomic Research (TIGR). A cotton orthologous gene was selected under criteria dependent on amino acid sequence length. (1) If the amino acid sequence length ≥ 100: the percentage of sequence identity ≥ 26%, percentage of positives ≥ 50%, and expected P-value ≤ 0.001. (2) If 30 ≤ amino acid sequence length < 100: then the parameters were set at 38%, 60%, and 0.001, respectively. According to those criteria, a total of 806 cotton ESTs and cDNAs

(http://polyploidy.tamu.edu/microarray) were identified as candidate cotton orthologous genes related to drought and/or cold response. On average, eight cotton EST or cDNA sequences were identified as having high sequence similarities with each cold- or drought-related query orthologous gene. A modified Featurama program (http://probepicker.sourceforge.net) was used to design cotton 70-mer oligos. Sequence lengths of 70 nucleotides with similar melting temperatures were selected within 1,000 nucleotides of the 3' end of EST or cDNA sequences (LEE et al. 2003). Each oligo represented a unique EST or cDNA sequence. A total of 1,536 70-mer oligos were synthesized, including 730 genes for chromatin proteins, cell-wall biosynthesis, cell cycle regulation, and signal transduction pathways. Oligos, with amino-linkers at the 5'-C6, were synthesized in Operon/Qiagen (Alameda, CA). Oligo sequences and their corresponding EST and cDNA accession numbers can be found on our website (http://polyploidy.tamu.edu/microarray).

Microarray fabrication and slide preparation: The oligos were delivered lyophilized in 384-well plates and were re-suspended in 20 µl 3x SSC printing solution to yield a 30 µM solution. Oligos were printed onto poly-L-lysine-coated slides (CEL Associates, Houston, TX) with 250-µm space between the center of spots using an OmniGrid Accent™ Microarrayer (GeneMachines, San Carlos, CA). Each slide contained two replicates of 1,536 features printed in 16 blocks. Each replicate contained eight blocks. The printing patterns for the two replicates within a slide were identical.

TABLE 7

Ninety-three drought and/or cold related genes used to design 70-mer cotton oligos

Species	Gene name	Accession number	Putative function	DR*	CR*	Reference**
A. thaliana	rd29A	D13044	hydrophilic protein	y*	y	(1)
A. thaliana	erd10	D17714	group II LEA protein	y	y	(1)
A. thaliana	rd17	AB004872	group II LEA protein	y	y	(1)
A. thaliana	rd20	AB039924	Ca-binding EF hand protein	y	nd*	(1)
A. thaliana	erd4	AB039928	membrane protein	y	y	(1)
A. thaliana	erd14	D17715	group II LEA protein	y		(1)
A. thaliana	rd19A	D13042	thiol protease	y		(1)
A. thaliana	rd22	D01113	unidentified seed protein	y		(1)
A. thaliana	FL3-5A3	AB044404	Putative cold acclimation protein	y	y	(1)
A. thaliana	FL6-55	AB050548	LEA 76 type 1 protein	y	nd	(1)
A. thaliana	FL5-77	AB050556	peroxiredoxin TPX1	y		(1)
A. thaliana	FL3-5J1	AB050557	gamma tonoplast intrinsic protein 2 (TIP2)	y		(1)
A. thaliana	FL5-1N11	AB050558	non-specific lipid transfer protein (LTP1)	y		(1)
A. thaliana	FL5-95	AB050576	rice glyoxalase I homolog	y	y	(1)
A. thaliana	FL5-2O24	AB050549	putative water channel protein	y	nd	(1)
A. thaliana	FL5-2G21	AB050560	reversibly glycosylated polypeptide-3 (RGP)	y	nd	(1)
A. thaliana	FL5-1A9	AB050542	nodulin-like protein homolog	y	y	(1)
A. thaliana	FL5-94	AB050561	enolase	y		(1)
A. thaliana	FL5-3J4	AB050562	heat shock protein dnaJ homolog	y		(1)
A. thaliana	FL5-3M24	AB050563	LEA protein SAG21 homolog	y	y	(1)
A. thaliana	FL5-2I23	AB050564	ascorbate peroxidase	y		(1)
A. thaliana	FL1-159	AB015098	HVA22 homolog	y	nd	(1)
A. thaliana	FL3-27	AB044405	cysteine proteinase inhibitor homolog	y	nd	(1)
A. thaliana	FL5-2I22	AB046991	DC 1.2 homolog	y	y	(1)
A. thaliana	FL5-1C20	AB050543	major latex protein type I homolog	y		(1)
A. thaliana	FL2-1H6	AB050565	*Brassica napus* jasmonate-inducible protein homolog	y		(1)
A. thaliana	FL5-2E17	AB050566	*Brassica napus* beta-glucosidase homolog	y		(1)
A. thaliana	FL3-3B1	AB050567	hypothetical protein	y		(1)
A. thaliana	FL5-3E18	AB050568	aquaporin homolog	y		(1)
A. thaliana	FL5-3A15	AB050569	fenitin	y	y	(1)
A. thaliana	FL2-56	AB050570	glycine-rich protein 3 short isoform (GRP35)	y		(1)
A. thaliana	FL5-2D23	AB050544	T20517 (EST) homolog	y		(1)
A. thaliana	FL3-2C6	AB050571	thioredoxin	y		(1)
A. thaliana	FL5-1P10	AB050572	putative photosystem I reaction center subunit II precursor	y		(1)
A. thaliana	FL2-5G7	AB050551	catalase 3 (CAT3)	y		(1)
A. thaliana	FL2-1C1	AB050573	cysteine proteinase homolog	y		(1)
A. thaliana	*DREB1A*	AB007787	EREBP/AP2 protein	nd	y	(1)
A. thaliana	FL2-5A4	AB050574	DEAD box ATPase/RNA helicase protein (DRH1)	nd	y	(1)
A. thaliana	FL5-90	AB050575	beta-amylase	nd	y	(1)
A. thaliana	FL5-3P12	AB050552	EXGT-A2		y	(1)
A. thaliana	RD28	P30302	Water channel	y		(2)
A. thaliana	rd19A, rd21A	AAM91778, AY072130	Cysteine proteases	y		(2)
A. thaliana	UBQ1	AY059080	Ubiquitin extension protein	y		(2)
A. thaliana	cATCDPK1, cATCDPK2	D21805, D21806	Ca^{2+}-dependent, calmodulin- independent protein kinases	y		(2)
A. thaliana	cAtPLC1	D38544	Phosphatidylinositol-specific phospholipase C	y		(2)
A. thaliana	Atmyb2	D14712	MYB-protein-related transcription factor	y		(2)
A. thaliana	ERD11, ERD13	D17672, D17673	Glutathione *S*-transferases	y		(2)
A. thaliana	cAtsEH	D16628	Soluble epoxide hydrolase	y		(2)
A. thaliana	cAtP5CS	D32138	$delta^1$-pyrroline-5-carboxylate synthetase	y		(2)
A. thaliana	*ERD6*	D89051	Sugar transporter		y	(3)

TABLE 7 (continued)

Species	Gene name	Accession number	Putative function	DR*	CR*	Reference
A. thaliana	*FAD8*	L27158	w -3 Desaturase		y	(3)
A. thaliana	*ADH*	X77943	Alcohol dehydrogenase		y	(3)
A. thaliana	*TCH4*	U27609	Xyloglucan endotrans-glucosylase		y	(3)
A. thaliana	*CBF1* (*DREB1B*), CBF2 (DREB1C), CBF3 (DREB1A)	U77378, AF074601, AB007787, AF074601, AB007788, AF074602, AB007789	Transcription factor		y	(3)
A. thaliana	*MEKK1*	D50468	Mitogen-activated protein kinase kinase kinase		y	(3)
A. thaliana	*MPK3*	D21839	Mitogen-activated protein kinase		y	(3)
A. thaliana	*PK19*	D42061	Ribosomal S6 kinase		y	(3)
A. thaliana	*CCR1, CCR2*	L04171, L04172	RNA binding protein		y	(3)
A. thaliana	*RCI1A, RCI1B*	X74140, X74141	14-3-3 Protein		y	(3)
A. thaliana	*PLC1*	D38544	Phosphatidylinositol-specific phospholipase C		y	(3)
A. thaliana	*TCH2*	AF026473, gi3123295	Calmodulin-related protein		y	(3)
A. thaliana	*TCH3*	P25071, gi3123296	Calmodulin-related protein		y	(3)
B. napus	*BN59*	U15604	ATPase (70-kDa subunit)		y	(3)
B. napus	*btg-26*	S77096	Aldehyde dehydrogenase		y	(3)
B. napus	*BnC24A, BnC24B*	Z22618, Z22620	60S ribosomal protein L13		y	(3)
C. plantagineum	GapC-Crat	X78307	Cytosolic glyceraldehyde 3- phosphate dehydrogenase	y		(2)
C. plantagineum	pSPS1	Y11821	Sucrose-phosphate synthase	y		(2)
C. plantagineum	pSS1, pSS2	Y11795	Sucrose synthases	y		(2)
Cotton	D19	M73751	LEA	y		(4)
Cotton	LeaA2-A	M83303	water-stress protectant protein	y		GeneBank
Cotton	Lea4-D9	M85261	putative desiccation protectant	y		GeneBank
Cotton	Lea4-A108	M86754	desiccation protectant protein	y		GeneBank
Cotton	LeaA2-D132	M83304	drought related	y		GeneBank
Cotton	D11	M81654	dehydrin	y		(4)
Cotton	D147b	M81655	dehydrin	y		(4)
Cotton	D113	M19406	LEA	y		(4)
Cotton	D29	M19388	LEA	y		(4)
Cotton	D34	M19389	LEA	y		(4)
G. max	SC514	X56139	Lipoxygenase	y		(2)
H. vulgare	pBAD	D26448	Betaine aldehyde dehydrogenase	y		(2)
H. vulgare	*blt801*	X81974	RNA binding protein		y	(3)
H. vulgare	*blt4.1, blt4.2, blt4.6, blt4.9*	X56547	Lipid transfer protein		y	(3)
H. vulgare	*blt63A*	Z23130	Translation elongation factor-1a		y	(3)
L. chilense	pcht28	L19342	Acidic endochitinase	y		(2)
L. esculentum	P31	M37150	Cytosolic copper/zinc superoxide dismutase	y		(2)
L. esculentum	SAM1, SAM3	Z24741, Z24743	*S*-adenosyl-L-methionine synthetases	y		(2)
M. crystallinum	pPPC1	X14587	Phosphoenolpyruvate carboxylase	y		(2)
M. sativa	*CIC, PRP2*	L22305, L37017	Proline-rich cell wall protein		y	(3)
P. sativum	*Sod 2* gene	M63003	Cytosolic copper/zinc superoxide dismutase	y		(2)
P. sativum	*Apx1* gene	M93051	Cytosolic ascorbate peroxidase	y		(2)
T. aestivum	PKABA1	AF519805	Protein kinase	y		(2)
T. aestivum	pMBM1	L07941	L-isoaspartyl methyltransferase	y		(2)
T. aestivum	*wcor518*	U73214	Proline-rich protein		y	(3)

* DR = drought related, CR = cold related, y = yes, nd = not determined, blank = unknown

** (1) = Seki et al. 2001, (2) = Ingram and Bartels 1996, (3) = Thomashow 1999, (4) = Basra 1994

After printing, slides with the DNA-side up were incubated at 80 ºC for one hour followed by UV-cross-linking at 100 mJ using 1800 Stratalinker (Stratagene, La Jolla, CA). Slides were exposed to high humidity by holding them face down over 37 ºC 1x SSC for 80 seconds followed by baking (face up) on 110~120 ºC heat-blocker for one minute. The slides were then treated with succinic anhydride in a "blocking step". Slides were quickly and evenly plunged up and down in a blocking solution (containing 9 g succinic anhydride dissolved in 500 ml 1-methyl-2-pyrrolidinone and 22.4 ml 1 M (pH 8) sodium borate added and mixed immediately before use) for 1 minute, followed by slow shaking (300 RPM) at room-temperature for 20 minutes. The slides were washed with H_2O twice each for 30 seconds, then briefly washed with 95% ethanol by plunging up and down several times. The slides were then dried by centrifugation for 5 minutes at 900 RPM. Treated slides were used immediately or stored in a humidity-controlled chamber (10~20% RH).

Plant materials and cold treatment: The plant materials used in this study came from cotton strain 'Tamcot Sphinx' (*Gossypium hirsutum* L.), an inbred line developed in the Texas Multi-Adversity Resistance Genetic Program for the improvement of resistance to pests and abiotic stresses (EL-ZIK and THAXTON 1996). Two hundred cotton seed were sterilized for 5 minutes in 2% bleach pre-cooled to 13 °C, then washed five times with sterilized water pre-cooled to 13 °C. The seed were then soaked in sterilized 13 °C water for 20 minutes for imbibition. Seed were then placed on moistened filter paper in covered Petri dishes and germinated in a growth chamber at 13 ºC in dark. When about

50% seed were germinated (in 13~15 days), that is, showing elongated radicals, germinated seed were collected and frozen using liquid nitrogen and stored at –70 ℃. For control, 200 seed were treated using the same procedure except that the temperature was set at 32 ℃ for sterilization, washing and germination.

RNA extraction and mRNA purification: Total RNA from cold-treated seed and controls was extracted pair-wise. Five grams of germinated seed were ground in liquid nitrogen, and transferred immediately into a Nalgene high-speed centrifuge tube containing 20 ml extraction buffer (200 mM Tris-HCl (pH 8.5), 1.5 % (w/v) lithium dodecyl sulfate, 300 mM lithium chloride, 10 mM EDTA, 1 % (w/v) sodium deoxycholate, 1 % (w/v) Tween 20). Just before use, we added 50 µl each of 10 mM dithiothreitol, 1 mM aurintricarboxylic acid, and 10 mM thiourea, and 2 ml 2% (w/v) PVP-40,000. After thoroughly mixing, the samples were incubated in –70 °C for 15 minutes, then thawed in 65 °C waterbath for about 1~2 minutes, and homogenized by mixing. The resulting materials were centrifuged at 3,500 RPM, 4 °C for 15 minutes. Supernatant was transferred to new tubes containing 1/30 volume of 3 M sodium acetate (pH 5.2) and 1/10 –20 °C absolute ethanol. After incubation on ice for 30 minutes, samples were centrifuged at 6,500 RPM for 20 minutes. Supernatant was transferred to new tubes, and 1/10 3 M sodium acetate (pH 5.2) and 1/3 isopropanol were added. After incubating at –20 °C for 2 hours, the tubes containing the samples were centrifuged at 8,000 RPM for 20 minutes. The pellets were collected and dissolved in 5 ml diethyl pyrocarbonate (DEPC) H_2O. To remove debris, the samples were centrifuged at 13,000

RPM for 5 minutes, and the supernatant was transferred to new tubes. To precipitate total RNA, 1/4 volume of 10 M lithium chloride was added and the samples were incubated at 4 °C overnight. RNA was collected by centrifugation at 12,000 RPM, 4 ºC for 20 minutes. The pellet was dissolved in 3 ml H_2O followed by adding 1.5 volumes of 5 M potassium acetate. The samples were incubated on ice for 2 hours. The total RNA pellet was collected by centrifugation at 12,000 RPM, 4 ºC for 20 minutes. The pellet was dissolved in 1 ml H_2O on ice, and 1/9 volume 3 M sodium acetate (pH 5.2) and 2 volumes cold absolute ethanol were added. The samples were incubated at -70 ºC overnight. The total RNA was collected by centrifugation at 13,000 RPM, 4 ºC for 20 minutes. After drying, the total RNA pellet was dissolved in 200 µl H_2O and stored RNA at -70 ºC freezer. During the extraction process, all solutions (except Tris-HCl solution, pH 8.5) were made up with DEPC H_2O when applicable, and all glass and plastic wares were treated with 0.1 % DEPC H_2O followed by a chloroform wash.

Total RNA was first treated with RNase-free RQ1 DNase (Promega catalog # M198A) to remove DNA contamination. Five hundred micrograms of total RNA were treated with 20 units DNase at 37 °C for 20 minutes, followed by phenol-chloroform purification and ethanol precipitation. The mRNA was then purified from total RNA with mRNA purification kits according to the manufacture's instructions (Invitrogen catalog # K1593-03).

Probe labeling, slide hybridization and data capture: The cDNA was synthesized and labeled with Cy3 and Cy5 using Amersham CyScribe Post-Labelling Kit following the manufacture's instructions (Amersham catalog # RPN5660), except that 1 μg mRNA was used for each reverse transcription reaction. We found that the mRNA amount used to synthesize the probe cDNA was critical. The probes should be excess during hybridization. For each slide, 1 μg mRNA of probe was used for each sample (cold-treated and control). Single-stranded cDNA and Cy3- and Cy5-labeled cDNA were purified using a purification kit (QIAGEN catalog # 28104) following the manufacture's instructions. Hybridization was performed in a total volume of 40 μl solution containing 6.5 μl 20x SSC, 4 μl 10% BSA, 28.5 μl Cy3- and Cy5-labeled cDNA probes, and 1 μl 10% SDS. The hybridization solution was heated at 98 °C for 2 minutes to denature the probes, and the hybridization was carried out at 65 °C for 15 hours. After hybridization, slides were washed using 2x SSC and 0.5% SDS at 37 °C for 4 minutes, 0.2x SSC twice at room temperature each for 2 minutes, and 0.05x SSC at room temperature twice each for 1 minute, respectively. Slides were dried by centrifugation at 850 RPM for 5 minutes and were scanned using GenePix4000B scanner from Axon Instruments.

A dye-swap experimental design was employed. Dye-swap experiments are a simple and effective design for comparing two targets directly, in which they use two slides but switch the color of the fluorescent dyes for the two targets. Dye-swap experiments were

repeated one time. Thus, a total of four slides were used for the same two biological targets.

Statistic analysis: the notation X_{ijmkg} was used to denote the observed value of gene G_g ($g = 1, 2, \ldots, 1536$) under target condition T_k ($k = 13$ °C or 32 °C) in replication R_m ($m = 1, 2$) labeled with dye D_j (j = Cy3 or Cy5) in slides S_i ($i = 1, 2, 3, 4$). After log transformation, $Y_{ijmkg} = \log(X_{ijmkg})$, ANOVA analysis was carried out using a linear model: $Y_{ijmkg} = \mu + S_i + D_j + R_m + T_k + G_g + AG_{ig} + DG_{jg} + RG_{mg} + TG_{kg} + e_{ijkmg}$, where μ represents the overall mean effect; S, D, R, T, and G represent main effects from the slide, dye, replication, target, and gene, respectively; the interaction terms SG, DG, RG, and TG represent gene by slide, dye, replication, and target, respectively; and e_{ijkmg} represents random error and was used to test for significance of main and interaction effects in this model. Due to confounding and /or aliasing issues involving the slide, dye, target, and gene terms, not all two-way and multi-way interactions are included in the model. The model residuals were assumed to follow normal distribution with a common variance, that is, e_{ijkmg} follows $(0, s^2)$.

The presence of differential expression in a microarray expression is represented by significant differences in 'T + TG' term for a particular gene. The following hypotheses were tested to determine whether a gene "g" has undergone differential expression between targets 13 °C and 32 °C RNAs:

H_0: $T_k + TG_{kg} = T_{k'} + TG_{k'g}$ (identical expression, i.e., no response)

H_1: $T_k + TG_{kg} \neq T_{k'} + TG_{k'g}$ (cold-response)

A standard t-test was used for the comparison based on the assumption of normality for the residuals. To control for multiple testing errors, both tests with Holm's sequential test for family-wise error rate (FWER) and Hochberg's false discovery rate (FDR) were employed. Holm's sequential method provides stronger control of the FWER below level α than Hochberg's method which controls FDR below level α (HOCHBERG and TAMHANE 1987; BENJAMINI and HOCHBERG 1995). The significance level was set at a = 0.05 level for this study. All oligos with significance at a = 0.05 were claimed as candidate cold-responsive genes.

Northern confirmation: We also assessed expression of certain genes using standard northern blots (SAMBROOK et al. 1989). Samples of total RNA from both cold-treatment and control were electrophorized in a 1% agarose gel containing 2% formaldehyde and then blotted onto membrane using 20x SSC. The membrane was pre-hybridized with Church buffer (CHURCH and GILBERT 1984) (0.25 M NaH_2PO_4, 0.25 M Na_2HPO_4, 2.5% SDS) at 65 °C overnight. For every candidate gene identified in the microarray experiment, a pair of forward and reverse primers was designed according to its corresponding cDNA or EST sequence. A DNA fragment was amplified by RT-PCR using mRNA prepared from cold-treated samples. About 50 ng of the DNA fragment

amplicon was labeled with a-^{32}P, denatured at 95 °C for 10 minutes, and then added to the Church buffer and hybridized to the membrane at 65 °C overnight. Following hybridization, the membrane was washed with 1x SSC and 0.5% SDS twice at room temperature for five minutes each, then with 0.2x SSC and 0.2% SDS twice at 65 °C for 20 minutes each. The membrane was used to expose Imaging Plate (FUJIFILM BSA-MS 2325), which was then scanned by a Fujifilm BAS-1800II machine.

To enable numerical comparisons between oligo-gene microarray and northern analysis, the fold-changes were also estimated from northern blots using a Fujifilm BAS-1800II machine. The northern fold-change was defined as the ratio of signal intensity after cold treatment versus control treatment in a defined area, which was defined as approximately equal to the band size of the up-regulated gene in a given blot. Background effects were removed.

Results

Microarray hybridization: A representative section of a microarray image is depicted in Figure 10. In the left panel, the slide was hybridized with cDNAs from the cold-treatment (Cy3-labeled) and the control (Cy5-labeled), while in the right panel, the labeling dyes were reversed.

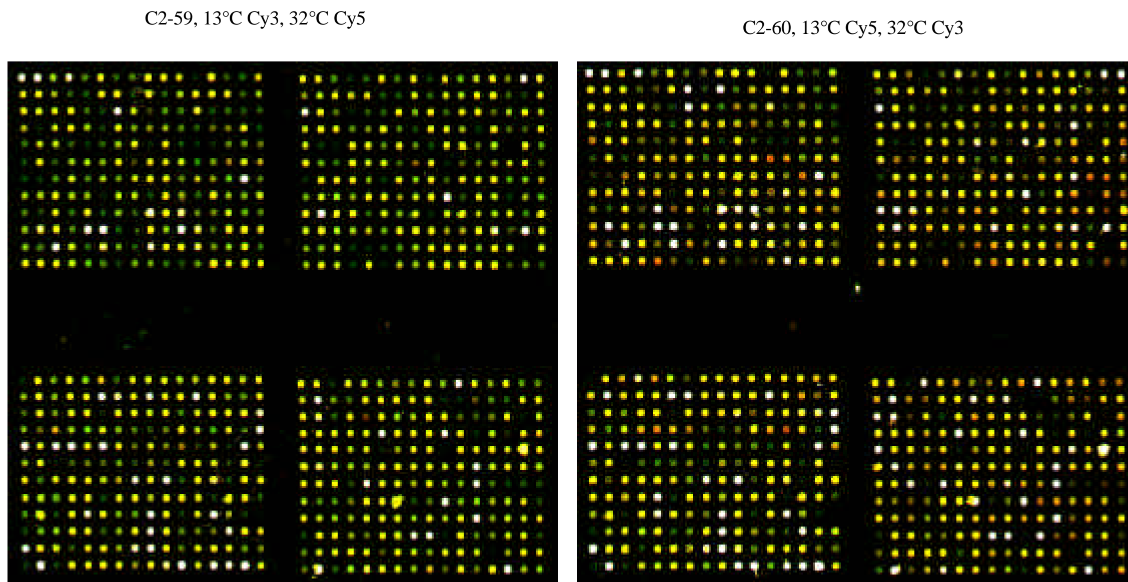

Figure 10: Microarray hybridization images obtained from one dye-swap experiment. In the left panel, probes from cold treatment and control were labeled with Cy3 (green) and Cy5 (red), respectively. A gene is up-regulated (green), down-regulated (red) or expressed at equal levels (yellow) under cold condition. In the right panel, probes were labeled with reciprocal dyes such that the colors of the images were switched from those in the left panel.

Four primary types of spots were found on a given slide, i.e., red, green, yellow, and white. The white spots resulted from high fluorescent intensity. The former two types represent genes that are up- or down-regulated by cold treatment depending on labeling, and the yellow type represents equally expressed genes. Even though bright spots could result from very high intensity of red or green only, we did not identify any genes from oligos showing bright spots. So, we believe the white spots also represent equally expressed genes.

ESTs and cDNAs responsive to cold temperature treatment: Significant effects were found by ANOVA for all components in our linear model except the interaction between arrays and oligos at $\alpha = 0.05$ level (Table 8). The background residual was depicted in Figure 11, which indicated relatively small effects due to hybridization background because most oligos had fitted values around zero. The mean squares for interactions between array and oligo, treatment and oligo, and replication and oligo within a slide were relatively small compared to other model components. So, we omitted the interaction effects in our model, and did further analyses to identify cold-related genes by Benjamini and Hochberg's FDR and Holm's FWER tests. The "fold-change" was defined as the ratio of hybridization intensities of the cold treatment divided by the normal-temperature control treatment. It was noticed that many oligos had significant fold-changes with the FDR test even though their fold-change values are small with absolute values less than two (Figure 12). This might indicate the sensitivity

Table 8

ANOVA analysis on microarray data

Sources*	SS	DF	MS	P value
S	395.26	3	131.75	0
D	18.81	1	18.81	0
T	89.25	1	89.25	0
R	24.60	1	24.60	0
G	34753.16	1535	22.64	0
SG	4713.13	4605	1.02	0
DG	314.29	1535	0.20	0.82
TG	425.98	1535	0.28	1.08E-13
RG	671.60	1535	0.44	0
E	2931.49	13824	0.21	
Total	44337.57	24575		

* S = slide, D = dye, T = treatment, R = replication
G = gene, SG = slide x gene, DG = dye x gene
TG = treatment x gene, RG = replication x gene
E = error

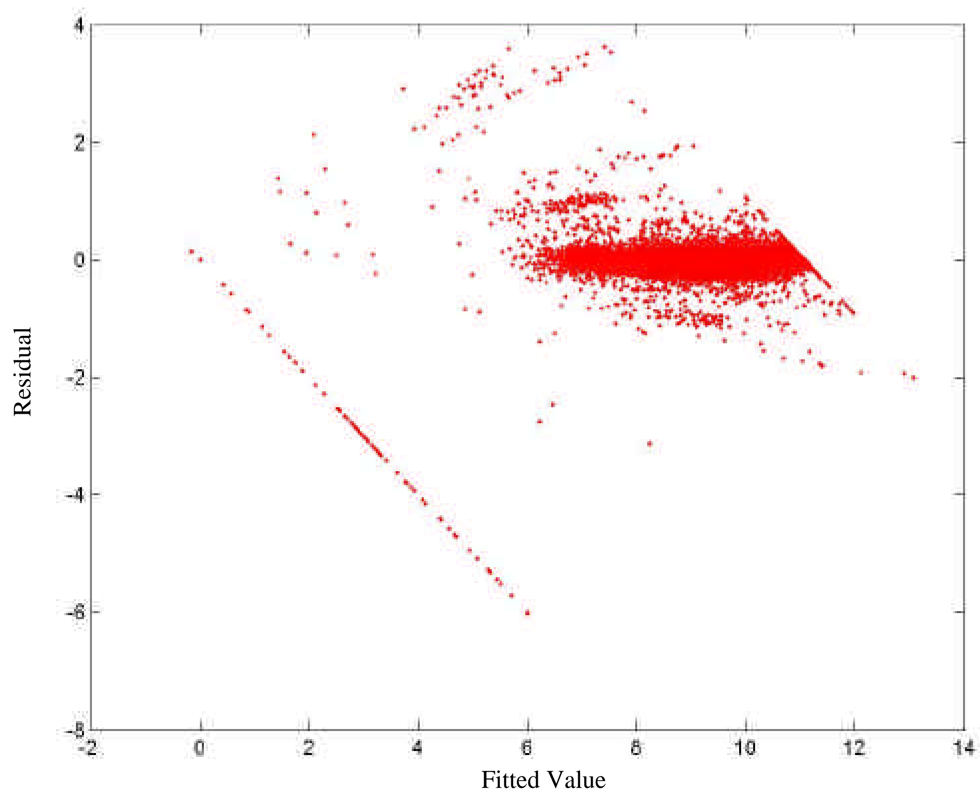

Figure 11: Residual distribution. Most oligos have fitted values around zero, indicating small residuals of slide hybridization.

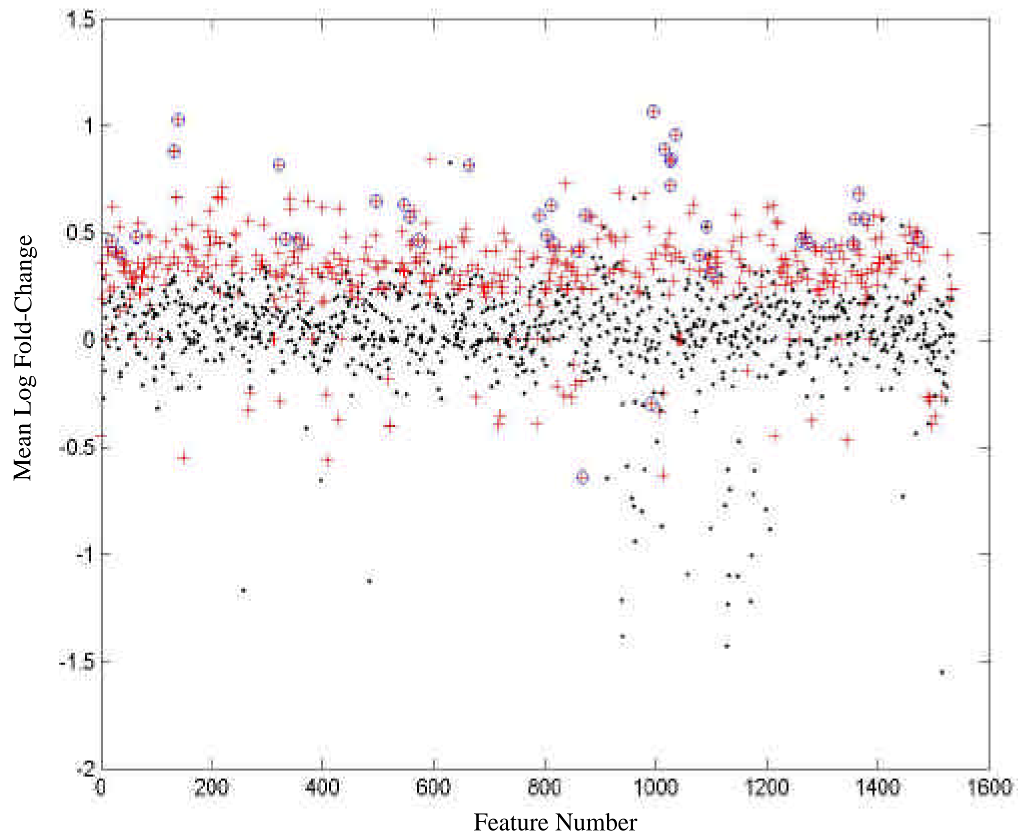

Figure 12: Significant genes detected in oligo-gene microarray experiment (a = 0.05). Significant genes are indicated by plus markers and circled plus markers, using Benjamini and Hochberg's FDR and Holm's FWER tests, respectively.

of statistical design and oligo-gene microarray in distinguishing gene expression levels. The FWER test uses more stringent criteria to declare significant differences than the FDR test. According to the FWER test, 38 cDNAs and ESTs were identified as cold temperature related genes (Figure 12, Table 9). Thirty-six of them were up-regulated with a range of 1.37~2.92, and only two down-regulated with fold-change values of 0.53 and 0.74, respectively. Sixteen out of the 38 identified cDNAs or ESTs (42%) resulted from the oligos designed according their homologies with reference orthologous genes.

Putative gene functions of 38 identified genes were listed in Table 10. BLASTx search indicated that putative proteins had homology to proteins of late embryogenesis abundant proteins, water-channel proteins, cell division related proteins, ripening-related proteins, chloroplast-related proteins, and heat-shock proteins.

Northern confirmation: According to microarray results, the expression levels for 10 of the 38 identified genes changed more than two-fold, whereas the others changed from 1.37- to 2-fold. To assess the microarray results, we did standard northern analyses. Primers (Table 11) were designed according to sequence information for each of the 38 identified ESTs and cDNAs. Double stranded cDNA probes were generated through RT-PCR using mRNA from cold treatment. Ideal probes were generated for only 17 of the cDNAs/ESTs. We did not succeed with the other 22 cDNAs/ESTs because either no PRC products were obtained or the product sizes were significantly different from the

expected sizes. The northern data and images were listed in Table 9 and depicted in Figure 13, respectively.

We compared fold-changes for all 17 genes with oligos and probes generated in microarray and northern analyses, and found that they were generally consistent. An exception was the EST TC3543, for which a fold-change of 1.7 was detected in microarray analysis, whereas northern blot analysis showed similar expression with a fold-change of 0.97. However, it was noticed the fold-change values were different (Table 9). With the convenience of replications in microarray experiments, small fold-changes less than 1.5 were readily detected. In duplicate northern analysis, it was impractical to identify those genes. Although measuring membrane radioactivity emission can help, it is difficult to control film exposure in northern blots, variation of which could easily cause bias on the measurement of fold-change. We believe microarrays are a more convenient means than northern blots to identify differentially expressed genes with small fold-changes.

TABLE 9

Changes in gene expression detected by microarray and northern blot analyses

Gene	MFC*	P value	NFC**	Concordance of analyses
AI054751	1.59	1.75E-05	***	***
AI054964	1.50	2.11E-05		
AI055397	1.61	1.80E-05		
AI726825	2.41	2.92E-06	1.27	Yes
AI727054	2.80	1.63E-06	1.66	Yes
AW587451	2.27	5.19E-06	1.87	Yes
AW587464	1.60	7.14E-07	1.22	Yes
AW587488	1.59	1.78E-05		
BF269690	1.91	3.13E-05	1.47	Yes
BF273236	1.89	6.41E-06	2.41	Yes
BF274167	1.77	1.29E-05	2.12	Yes
BF275215	1.59	1.14E-05		
BG440630	2.26	8.57E-06	1.50	Yes
BG445739	1.79	5.89E-06		
BG446403	1.63	5.70E-06		
BG446723	1.87	1.50E-06	1.11	Yes
BG446904	1.56	4.31E-06		
BM359350	1.51	7.34E-06		
BM359622	0.53	1.09E-05	0.90	Yes
BM359908	1.79	4.04E-06		
petB	0.74	1.92E-05		
psaB	2.92	1.07E-06	8.12	Yes
rpl20	2.44	8.32E-06		
rpoB	2.30	4.40E-06		
rpoC1	2.06	4.62E-06		
rpoC2	2.34	5.88E-06		
rps2	2.61	2.20E-07		
TC3447	1.48	8.36E-06		
TC3543	1.70	3.31E-05	0.97	No
TC3625	1.37	1.21E-07	1.53	Yes
TC5122	1.59	1.32E-05		
TC5193	1.57	7.14E-06		
TC5396	1.55	5.64E-06		
TC5737	1.56	3.10E-05		
TC5764	1.76	2.55E-05	1.90	Yes
TC5856	1.98	3.56E-06	1.53	Yes
TC5979	1.77	9.22E-07	1.21	Yes
TC7484	1.61	5.41E-07	1.64	Yes

* MFC = oligomicroarray fold-change (treatment/control)
** NFC = northern fold-change (treatment/control)
*** blank = not determined

TABLE 10

Annotation of cold-responsive genes

Gene ID	Annotation
AI054751	putative protein, homology to late embryogenesis abundant protein family
AI054964	putative protein, homology to lactoylglutathione lyase in Arabidopsis and gi:6323639 in yeast which is Regulated by HOG (high osmolarity glycerol)-MAP (mitogen-
AI055397	putative protein, homology to pectinesterase and ripening-related protein
AI726825	putative protein, homology to phytocyanin/early nodulin-like protein
AI727054	putative protein, homology to heat shock protein
AW587451	putative protein, homology to cell wall surface anchor family, knob-associated his-rich protein, neuromodulin, and growth-related protein
AW587464	putative protein, homology to class I heat-shock protein (chaperonin), RuBisCO subunit binding-protein alpha subunit, chroloplast precursor (60 kDa chaperonin alpha
AW587488	putative protein, homology to serine carboxypeptidase
BF269690	putative protein, homology to SET-domain transcriptional regulator family
BF273236	putative protein, homology to cell division-related protein and cyclin-dependent protein kinase
BF274167	hypothetical protein, homology to putative ORF or hypothetical protein
BF275215	putative protein, homology to RNA-binding protein and heterogeneous nuclear ribonucleoprotein (hnRNP)
BG440630	hypothetical protein, homology to hypothetical protein
BG445739	putative protein, homology to myb-related transcription factor
BG446403	putative protein, homology to MAP kinase kinase
BG446723	putative protein, homology to nucleosome assembly protein
BG446904	putative protein, homology to high mobility group (HMG) protein, nonhistone chromosomal protein, DNA-binding protein
BM359350	putative protein, homology to cytosine methyltransferase, sugar hydrolase and ubiquitin associated protein
BM359622	putative protein, homology to Histone H2A
BM359908	putative protein, homology to Histone H3
petB	putative chloroplast protein, high homology to chloraplast cytochrome B6
psaB	putative chloroplast protein, high homology to chloraplast photosystem I P700 apoprotein A2
rpl20	putative chloraplast protein, high homology to chloroplast ribosomal protein L20 and mitochondrial ribosomal protein L20 in human
rpoB	putative chloroplast protein, high homology to RNA polymerase beta subunit
rpoC1	putative chloroplast protein, high homology to RNA polymerase beta subunit-1
rpoC2	putative chloroplast protein, high homology to RNA polymerase beta subunit-2
rps2	putative chloroplast protein, high homology to ribosomal protein S2 and putative ribosomal protein S2 from chromosome 10 chloroplast insertion in rice
TC3447	putative protein, homology to 26S proteasome regulatory particle triple-A ATPase subunit2b
TC3543	putative protein, homology to ubiquitin family
TC3625	putative protein, homology to HSP70 binding protein and tetratricoredoxin
TC5122	putative protein, homology to membrane water channel protein aquaporins
TC5193	putative protein, homology to Guanine nucleotide-binding protein beta subunit-like protein and WD-40 repeat auxin-dependent protein ARCA
TC5396	putative protein, homology to 14-3-3 protein, regulate intracellular localization of transcription activator RSG in tobacco
TC5737	putative protein, homology to myb family transcription factor
TC5764	putative protein, homology to 14-3-3 protein
TC5856	putative protein, homology to 26S proteasome regulatory particle triple-A ATPase subunit4-related protein
TC5979	putative protein, homology to histone deacetylase related protein
TC7484	putative protein, homology to early drought induced protein gi:21314337 in rice and HMG protein

TABLE 11

Primers for generating the probes used in northern analysis

Gene ID	Froward primer sequences	Reverse primer sequences	Reference gene ID	Oligo sequences
AI054751	tttttagcaagtcaacaaaattga	ttgatagcaggcaaaggatg	AB050563	ctccgccagacatatcttcccctgttttcttggccaccgtagcagcgctccttcccgctcctcctcttat
AI054964	agaacaccctgcatttcacc	tgactttaaatcacacgaagaagg	AB050576	ctttcacctctctagaacaccctgcatttcaccttcttcactcccattttcttatatctcgaaaccccat
AI055397	cctcatcttcacaccatggac	catgctgcaatggcacttat	AB046991	accacaacatatcccaaattatgttaccagtccctctcagtctatgcgagcagcatcagcaccagcccca
AI726825	ttgggttttgcaccctaaag	actgtgctggacctggagtt	AB050542	aaagccacccacaggatcatctccggttcctgctctggctcttggtcctattgcaaagcctcctacagca
AI727054	gaacaaaatccccaagcaga	aataggggcaccttttggag	AB050562	aaaattggaaaaaaaagaacaaaatccccaagcagaagcagccaaaaacgggggcaaattgaagaatggg
AW587451	cctgcaaggtctcgagtttc	atcctccactggcactttgt	N/A	ttggcacctctgatcctcaaacaacagcaaaggatgagaaaagcagcagttcgagtcagtcccacaacaa
AW587464	ccaaaaacccagaaaccaaa	tgcacccgaagtaacagtca	N/A	acactctctgcctgcctcccccttatccactctttcccaaaaacccagaaaccaaaacaaagcctttatct
AW587488	caagcgctttcttcttcctc	ccagtgatagcagcaaccaa	N/A	ttatctcactgcttcaacaactcctctcttccccaaagacgccctccccaccaaatctggctaccttcct
BF269690	caaagtttaacacagctgaagacaa	tccgaagaaccggaatcttat	N/A	taaggagtcaaagtttaacacagctgaagacaaacaggttcctccaaaatcgcacccccctgatgaagaca
BF273236	tgcagactttggtctggcta	cttctcactccggctctttg	D21839	accagctttagctctcatggagaccttactttccattgaccctgctggtcgtggatctgcagcttctgca
BF274167	tccgacaagtccagcctact	ggtttttgagggcacaacat	N/A	acagtccgacaagtccagcctactctcctaccagcccctcatattctccaacctcaccaagctatagccc
BF275215	tcctcaaattccatttctttgg	acgcagtgatgctactcatgtt	L04171	gcaaaacctcctcttctgtttccgttgtcatcgtttctcatctcagatcttaattttcctcccattttct
BG440630	agtcccacttcacccagcta	gcatgccttatcgtttactcc	N/A	ttattccccttcaagcccaacatacagtcctagcagcccatacaactctggagtgagcccagactacagt
BG445739	cggtctccatgctgtgataa	atttccagccatgttccttc	D14712	gagtcaaaattccgtgggctcctcctcttcctctgcttctgcttcttcttcctgggctccacagcacact
BG446403	tgaagaagggaaagggaattg	acacagtgtatgggccacaa	D50468, D21839	agggaattgtaaaccctaatttgaagctctctcttcctcctccagccgagatctccttcgccaaatttct
BG446723	ccctttcaaaacacccttcc	tggcattctttgagccttct	N/A	ccgatggaactactgatgaagctgccatggaccaacgagatgataaagctgccgtacagaaaggagttcc
BG446904	agtccaaatcccagctcctt	ttccacttctcaccacaagc	N/A	gtcaaaacccaaatcgaagctgaagaggaaaacaaaaatcgatgctaaaatgcctaagaaaccccccact
BM359350	ttggggcttcccctatgtat	tacgaagccctcagcttctg	N/A	gtatattttatttcttttcctccaccggtaacttctttgtcagaactcgtcgacgatcgccgctgctgcc
BM359622	acctgctaccagcaatggac	cttctccgcggtcctctt	N/A	tgctcgccgtcaggaatgacgaggagctcggcaagctgctggccggcgttaccatcgcgcacggcggcgt
BM359908	cgacctcctcctccctagtc	tggatgtccttgggcataat	N/A	ccgaccttcggttacagtcctccgccgcctccgccctgcaggaggccgccgagtcctacctcgtccggcc
petB	tcaggcgattgcagatgata	gggaccagaaataccttgctt	N/A	acctcttcttacggccgtctttatgttaatgcatttcctaatgatacgtaagcaaggtatttctggtccc
psaB	ttatgcgttcatagcgcaag	gtggaggcaatcaagaaagc	N/A	tgattcgctggaaagataaaccagtagctctttcaatcgtgcaagcaagattggttggattagctcactt
rpI20	tcggaggcgtagaacaaaac	tcgaaattgtataaagacaactcctat	N/A	agaaaaagagattttcgccgtttatggatcactcgaataaatgccgtaattcacgaaatgggggtattct
rpoB	ccaaggtcctgaaaggatca	ccggagcatcttctggttta	N/A	aaccagaagatgctccggaatcttttcgattacttgttcgagaactacgatctttggctctggaactgaa
rpoC1	acccatgagggatggtcata	tgaacaggcttgagaaaaacc	N/A	cgaaccacagttggtcatatttctttttatcgagaaatcgaggaagctatacaaggtttttctcaagcct
rpoC2	aatcgatgaaaacggaataattt	atgttggtatgttgccgtga	N/A	gggaccggattcaacaaaggattggtgcattgttcacggcaacataccaacattattttggaaaaaaaaa
rps2	tggaacatcgatttggaaga	tagctagaacggccctcaca	N/A	acaaattgtaatcccgatctcgcggatatttctattcccgcaaatgatgacgctatagcttcaattcgat
TC3447	tgggaacaacttgaatccaga	gattaacgaggcaggtgagc	N/A	cgaatcaaagccatccaattggttcagcaactccaacatggtcctctgaatttcacgttcaccaccggaa
TC3543	tcgctattcagtttggctttg	cctgcgacgattttggtatg	AY059080	tctcttccttcagttttcttctgcctctctctttcattcgagagcctaaaaccctctctctccttcctca
TC3625	catcagccgtgatgttcttg	tgtgatggatgcttcaaagg	AB050571	tgtgcctcagctttcttctgttgcctctgacgttcactcctttttagttccctttctttccgcaggcgtt
TC5122	tccagcgatatctccacaca	cgagtgcacttgagggaaag	AB050557, AB050568	gcagagaaaacagtgtaaacaagcacaaaagtcccaattatctcagcaccaagaccatcacccttagtgt
TC5193	ggctcaatgaaaacctccaa	gtccatcatcctttggcact	N/A	actgtaaagcttctttccttcagccaaatcccacaacaaaataacaccatcttttccgccactagcacac
TC5396	atcatcggcccttcttcttc	ggaggagttgcatgatgagg	X74140	catcggcccttcttcttcaacgcaatcatcgcctcccacttctcccaccccctcaaaactccctcccgaa
TC5737	acccccaataaagcttgaaa	gggggaagagaaaaagaagc	D14712	tcacaacatggaggtcttcccatggcttcctttgcttctttttctcttcccccctccccccctctttggtt
TC5764	cctcgctgtggaaagaaaaa	gagagagacagagcccgtga	X74140	ctgcacaggatattgctctcggtgacctagcgccaactcacccaattaggctgggacttgctcttaactt
TC5856	gccctcactcaagaaacctct	cctatcagcctcgcactttc	N/A	gaagacgctgtacggcgccgaaatgccattgcggagtaccgaaagaaactccttcaacacaaggagtacg
TC5979	caaacatctctcccccaaaa	gacactaacgcgatccacaa	N/A	agcagcagcaacaggaagctcctcctcttcctcctcctcgctttcttcttcgctagacataccaaaatca
TC7484	aaatattaaaatgcagatccacca	tgaagaaacggaaaaagaagga	N/A	atcgctttttatcaaccctagcctttgcttcatatggttccttctcctcctttggcaactttctccacct

N/A = the oligo was designed not based on a reference gene

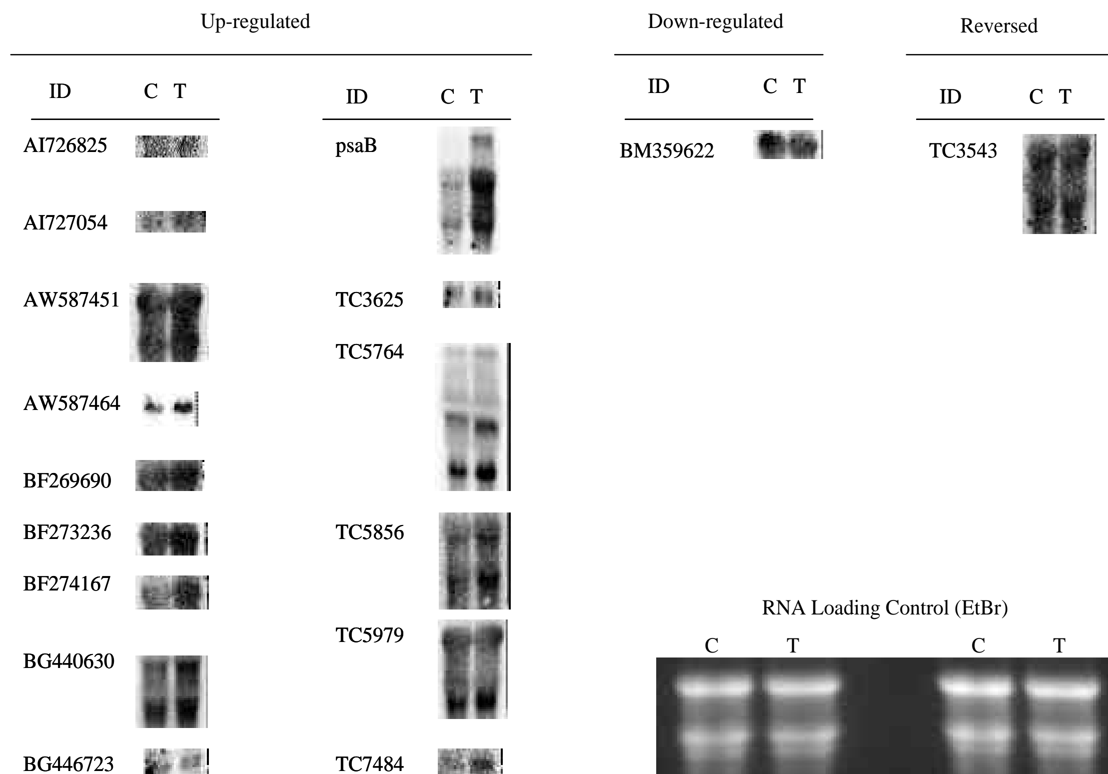

Figure 13: Northern blot images. C = control, T = cold treatment.

Discussion

Gene conservation and model organism application: Eukaryotic plants share the same origin. During evolution and speciation processes, gene structures and functions may diverge among species. However, some genes may be partially or wholly conserved in terms of structure and(or) function. Environmental stresses are generally common for all plant species. During evolution process, environment-stress related genes might face similar selection among plant species. Horvath et al. (3002) found genes for cell division, stress response and shoot growth were conserved among distantly related plant species. Jaglo et al (2001) reported that the CBF cold-response pathway was conserved in *Arabidopsis*, *Brassica*, wheat, rye, as well as in tomato. This research demonstrates that some genes related to cold response of *Arabidopsis* have conserved sequence compositions in cold-related genes of cotton, and that those genes might have conserved functions. Forty-two percent of the cold-related cotton genes identified in cotton were detectable based on homology information from *Arabidopsis* and other plant species.

Genomics of agronomically important crops such as cotton, wheat, and maize, lag far behind *Arabidopsis*. How to harness genomic studies of *Arabidopsis* for improvement of crop genomics is a pressing need. In terms of stress-related studies, *Arabidopsis* stress-related genes can be used directly in *Arabidopsis* microarrays to identify conserved stress-related genes and test expression in other plant species. A different strategy is to

identify candidate stress-related genes in the target species based on sequence similarities to *Arabidopsis* stress-related genes, and then create microarrays of endogenous genes for expression analysis. We believe the second approach is better because it allows for evolutionary gene sequence changes. Horvath et al. (2003) used *Arabidopsis* microarrays to study differentially expressed genes involved in shoot growth and development in distantly related species. They found that homologs showed greater variance in nucleic acid levels than amino acid levels and that different categories of genes exhibited different hybridization efficiencies to *Arabidopsis* genes on microarray. Microarray experiments based on target genes and probes from the same species will facilitate hybridization and thus foster more reliable and informative results. For many crop species, extensive sequence data from cDNAs and/or ESTs are available in the public domain. For example, current cotton data include sequences of about 16,000 cDNAs and ESTs. Developing microarrays for a given plant species should not be a big problem, especially for oligo-gene microarrays, since these require only gene-specific oligos, not cDNA or EST collections. This research demonstrates that the 70-mer oligo-gene microarray is an attractive alternative to cDNA amplicons for gene expression analysis. In addition, the second approach allows the researchers to advance genomic researches on species of interest from genomic studies of any other species wherever conserved or homologous sequence information is available. We designed our cotton microarray to study stress-related genes and some other agronomic traits using the cotton database and annotation information from *Arabidopsis* and some other plant species.

Candidate gene isolation approach for quantitative traits: The candidate gene approach has been used to identify many genes. However, most of the genes identified in this manner exert major effects on qualitative traits. For many complex traits that involve many genes each with a relatively small effect, the candidate gene approach is thought to be relatively inappropriate for gene identification and cloning. Based on sequence information from homologous genes we identified cold-related genes using the candidate gene approach and microarray analysis. Of the 38 genes identified, 42% were derived from reference orthologous gene information in *Arabidopsis*, whereas ten cotton genes were identified as drought-related genes over the last decade. The results indicate that it is feasible to combine the candidate gene approach and microarray technology to study agronomically important complex traits.

In this research, 16 of 93 candidate orthologous genes resulted in the identification of homologous cotton genes. The numbers indicate some differences in cold-responsive genes or systems among cotton, *Arabidopsis* and other species. It seems reasonable from an evolutionary stand point of view, duplicated genes could diverge during evolution to have differential expression patterns (ADAMS et al. 2003). However, some other factors may also be important. First, most of the candidate orthologous genes (INGRAM and BARTELS 1996; THOMASHOW 1999; SEKI et al. 2001) are drought-responsive genes, not all of them are cold-responsive. Although there are overlaps of induced genes during drought and cold stresses, each stress may be related to a subset of unique inducible genes. Indeed, none of the ten cotton drought-inducible genes was found to be cold-

inducible in this research. Cold-responsive genes such as wsc120 in wheat (HOUDE et al. 1992) and cas18 in alfalfa (WOLFRAIN et al. 1993) have been demonstrated to be responsive to low temperature, but nonresponsive to drought stress. Second, many candidate orthologous genes were identified from leaf tissues treated at 4 °C. Our research was conducted on seed germinated at 13 °C. Differential gene expression patterns were expected between leaves and germinated seed. Fowler and Thomashow (2002) reported that more than 50% of the cold-responsive genes in *Arabidopsis* were up or down regulated transiently in response to low temperature. It took about 15 days for cotton seed to germinate at 13 °C. The genes identified from this research represent those that show long-term effects on gene expression in response to low-temperature treatment.

Putative functions of identified genes: There are three groups of genes that could be identified during a differential cold-response screening process, but it is hard to tell which gene belongs to which group: (1) genes involved in signal transduction response to cold treatment; (2) genes encoding proteins or enzymes that result in cryoprotectants to protect plants from cold damage; and (3) genes required in normal metabolism but up- or down-regulated during cold stress. The genes identified in this research showed homology to late embryogenesis abundant proteins, cell division related proteins, ubiquitin protein family, histone deacetylase related proteins, chloroplast-related proteins, MAP kinase, myb-related transcription factor, water-channel proteins, and heat-shock proteins, *et cetera*. Cold-responsive genes with similar functions have also

been identified in other plant species (SEKI et al. 2001; FOWLER and THOMASHOW 2002, KOLLIPARA et al. 2002; XIONG et al. 2002). Thus, the candidate cotton genes may have functional importance during cold stress. For example, EST AI054751 showed homology with LEA proteins. Cold temperature and freezing can lead to cell dehydration, which can cause effects similar to drought or seed dehydration. Therefore, some cold-responsive genes can also be induced by dehydration. ESTs AI727054, AW587464 and TC3625 had homology with heat shock proteins. Cold stress can cause changes in membrane composition and structure. Some small heat shock related proteins protect plants from chilling injuries (SABEHAT et al. 1998), and heat shock proteins might also help stabilize membrane lipid phase (TOROK et al. 2003). EST TC5979 showed homology with histone deacetylase related proteins. We expected TC5979 might be involved in the CBF cold-response pathway because C-repeat binding factor 1 (CBF1) was reported to interact physically with a histone acetyltransferase (STOCKINGER et al. 2001). Similarly, it seems that EST BG446403 might be involved in cold stress signal transduction because it has homology with MAP kinase kinase, which is involved in signal transduction during response to stresses (SEO et al. 1995, JONAK et al. 1996). EST TC3543 might be involved in protein degradation because it has homology with the ubiquitin family. A significant proportion of the cold-responsive genes identified in this research were identified as seven chloroplast genes. Short-day photoperiod has been reported as stimuli to trigger cold acclimation in woody perennials (WEISER 1970; SAKAI and LARCHER 1987). Photooxidation and photoinhibition often occur during chilling (LAMBERS et al. 1998). Chloroplasts have also been demonstrated to be involved in

cold-tolerance. *Arabidopsis* cold-regulated gene *COR15* encodes a chloroplast-targeted polypeptide and has potent cryoprotective activity *in vitro* (LIN and THOMASHOW 1992 a, b). In cotton, gene petB encodes cytochrome B6, psaB encodes protein photosystem I P700 apoprotein A2, genes rpL20 and rpsS encodes ribosomal proteins, whereas genes rpoB, rpoC1 and rpoC2 encode proteins for RNA polymerase subunits. In this research, seed were germinated in dark. How these chloroplast genes affect cotton cold tolerance is not clear. However, it is expected that some chloroplast genes are actively involved in cotton cold-responsive pathways.

CHAPTER V

SUMMARY

Part I: Wide-cross whole-genome radiation hybrid (WWRH) mapping of cotton genomes

Cotton (*Gossypium* spp.) is the world's most important textile fiber crop. Eight 13-chromosome genomic groups (A, B, C, D, E, F, G, and K) have been identified in the *Gossypium* genus. Most cultivated cotton, however, evolved from a tetraploid cotton and thus contain two 26-chromosome AD genomes. Each AD genome contains about 3160 Mb DNA. Genome maps are highly desirable for integrative genomics, map-aided gene cloning, and marker-assisted breeding, but current maps of the cotton genome are incomplete. For example, the number of linkage groups in available public linkage maps of tetraploid cotton still exceeds its gametic chromosome number (26), and numerous linkage groups have not been assigned to chromosomes. Among the factors that have hindered cotton genome map development are [1] a very high rate of recombination in cotton, [2] nonrandomness of crossover distribution, [3] reliance on genome-wide marker development strategies, [4] incomplete genome coverage with hypo-aneuploids suited to physical mapping, [5] under-utilization of molecular cytogenetic physical mapping, and [6] relatively low levels of *intra*-specific molecular marker polymorphism within the two cultivated tetraploids, *G. hirsutum* and *G. barbadense*. Improved resolution and integration

of cotton genome maps are desirable. Many methods have been suggested to increase map resolution. One of them is radiation hybrid mapping, which has been widely used in the genome mapping of human and certain other animal species, but little used in plant species. Radiation hybrid (RH) mapping and whole genome radiation hybrid (WGRH) mapping of human and animal genomes have relied on panels of multiple *in vitro* radiation hybrid cell lines. In RH mapping, each panel is targeted at a single chromosome, whereas in WGRH mapping, each panel is targeted at the entire genome. The difficulty of finding appropriate recipient cell lines to rescue irradiation-fragmented plant chromosomes has been a major obstacle in the application of *in vitro*-based (WG)RH mapping in plant species. In this research, a wide-cross whole-genome radiation hybrid (WWRH) mapping method was developed, which successfully mapped genomes from two cotton species, *Gossypium hirsutum* (L.) and *G. barbadense* (L.). We created two *in vivo* WWRH mapping panels, one (N = 93) used the genome of *G. barbadense* (n = 26) to rescue radiation-segmented genomes of *G. hirsutum* (n = 26) introduced via 5-krad γ-irradiated pollen, the other (N = 92) used the genome of *G. hirsutum* (n = 26) to rescue radiation-segmented genomes of *G. barbadense* (n = 26) introduced via 8-krad γ-irradiated pollen. Marker retention in both panels was random along a given chromosome, among chromosomes and between A and D subgenomes according to tests on SSR markers from linkage group 9 (LG-9) in the 5-krad panel and 31 SSR markers from four chromosomes of two different genomes in the 8-krad panel. Marker retention frequencies were relatively high in comparison with those in human and certain animal species, with averages of 89% for SSR markers on LG-9 in the 5-krad panel and 76% for 31 SSR markers on chromosomes 10, 12, 17, and 18 in the 8-

krad panel. The 8-krad irradiation resulted in more retention patterns, lower marker retention frequencies and larger distances between markers than the 5-krad irradiation. The marker distance correlation coefficient between the traditional linkage map and WWRH maps varied for different chromosomes with 0.49 for LG-9 in the 5-krad panel, 0.66, 0.71 and 0.96 for LG-9, chromosome 10, and LG-13 in the 8-krad panel. Both panels were readily used to construct WWRH maps according to the RHMAP program. Syntenic groups were identified and WWRH maps were constructed for chromosome 17 from both panels using the RHMAP program. It was demonstrated that (1) WWRH mapping works in cotton; (2) WWRH mapping complements traditional linkage mapping, especially in genome regions with relatively high or low recombination frequency by ordering co-localized markers or detecting synteny between linkage groups, and assigning markers and/or linkage groups to chromosomes in linkage maps; (3) The WWRH mapping panel complements traditional cytogenetic aneuploid stocks; (4) The 8-krad panel complements the 5-krad panel by offering higher rates of chromosome breakages, lower marker retention frequency, and more retention patterns; (5) Analogous applications seem plausible for other species, particularly those that are functionally polyploid, and may be especially useful in those that are relatively recalicitrant to linkage mapping due to genomic complexity, apomixis, polysomy, hybridity, cultivar-specific interests, and/or long generation times.

Part II: Identification of cotton (*Gossypium hirsutum* L.) genes responsive to cold temperature through 70-mer oligo-gene microarray analysis

Plants grow in a highly variable environment and experience various environmental stresses throughout their life cycles including biotic and abiotic stresses. The most significant abiotic environmental stresses on plant species worldwide are drought, cold and salt stresses. Plant tolerances to environmental stresses are often quantitative traits controlled by many genes. Changes in gene expression in response to environmental stresses have been studied in many plant species. Different stresses often induce the expression of a set of unique and common genes. Even though forward genetics has successfully identified some stress-related genes, more and more genes are being isolated through reverse genetics approaches such as high-throughput microarray techniques. Cold stress is among the most important abiotic stresses in cotton production. Cold stress at early developmental stages often renders cotton susceptible to fungal infection and many other environmental stresses. Increasing cold tolerance could expand the cotton growing season, increase tolerance to other stresses such as seedling diseases, and subsequently increase fiber yield and quality. Cold tolerance is a quantitative trait in cotton, but in contrast to hundreds of cold-related genes or ESTs identified in model plant species *Arabidopsis*, no cold-inducible genes have been isolated in cotton. Studying cotton genes related to cold-tolerance will advance our understanding of cold-responsive mechanisms and facilitate cold-tolerant cotton cultivar improvement. To benefit cotton stress tolerance study from *Arabidopsis* genomic resources, a candidate gene identification approach was

employed in this research to isolate cotton cold-responsive genes using 70-mer oligo-gene microarray analysis. Eight hundred and six 70-mer oligos were designed to represent cotton cDNAs and ESTs which showed homology to or had high sequence similarity with orthologous genes identified as drought and/or cold stress related genes in *Arabidopsis* and several other plant species. Cotton oligo-gene microarrays containing 1,536 features (including genes encoding chromatin proteins, cell-wall biosynthsis, cell cycle regulation, and signal transduction pathways) were developed. Overall, 38 cDNAs or ESTs were identified as cold-responsive genes, of which 36 were up-regulated with a fold-change range of 1.37~2.92 and two were down-regulated with fold-changes of 0.53 and 0.74, respectively. The identified candidate cotton genes encode putative proteins with homologies to late embryogenesis abundant proteins, water channel proteins, cell division related proteins, chloroplast related proteins, and heat shock proteins. This research demonstrated that (1) Using microarray technology in combination with the candidate gene identification was feasible and applicable to the study of stress-tolerance traits; (2) Some cold-responsive homologous genes are conserved between cotton and *Arabidopsis*. Orthologous *Arabidopsis* genes can be used for identification of candidate genes in cotton, i.e., the genomic information from model plant species *Arabidopsis* can benefit genomic researches in other non-model plant species; (3) 70-mer oligo-gene microarray is an attractive alternative to cDNA amplicons for gene expression analysis.

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