COTTON INCORPORATED

To: Participants at the August 20, 2003 Meeting in Austin Texas, Carlos Avila, Al Bell, Peng Chee, Richard Davis, Nilesh Dighe, Brian Gardunia, Doug Hinchcliffe, Johnie Jenkins, Don Keim, Jeff Klingenberg, Andrew Kloek, Randall McPherson, Edina Moresco, Gerald Myers, Robert Robbins, Phil Roberts, Mike Robinson, Martha Schlicher, Wayne Smith, James Starr, Sallinana Stetina, Mac Stewart, Bob Stipanovic, Peggy Thaxton, David Weaver, Tom Wofford, Zhangyou Xu, Lawrence Young, and Hongbin Zhang

From: Bob Nichols, Forest Robinson, Dave Stelly, and Roy Cantrell

Subject: Report of the Meeting, "Breeding Cotton for Resistance to Nematodes"

Date: December 17, 2003

CC: Charlie Cook, Roy Creech, Lloyd May, Kathy McLean, Hal Moser, John Mueller, Don Panter, Frank Shotkoski, Ted Wallace

Summary

Objectives

The meeting focused on identifying, characterizing, and locating genes that might be used to develop germplasm with host plant resistance (HPR) against root-knot (*Meloidogyne incognita*) and reniform (*Rotylenchulus reniformis*) nematodes. The meeting also began addressing the task of transferring germplasm and necessary breeding technology to commercial planting seed companies to enable them to develop commercial cotton (*Gossypium hirsutum)* cultivars with resistance to these nematodes. The meeting's goals were to identify the specific technologies needed and to develop efficient research plans to provide them.

Root-Knot Nematode (RKN) – Resistance Genes

Screening of *G. hirsutum* germplasm suggests that RKN resistance genes are not uncommon. About 25 years ago, Ray Shepherd, a USDA-ARS breeder, developed a highly RKN resistant line, 'Auburn 623' (Shepherd 1974a and 1974b) from a cross of two moderately resistant *G. hirsutum* genotypes (Clevewilt 6-8 and Mexican Wild = USDA accession TX 2516); Shepherd subsequently crossed Auburn 623 with the Fusarium wilt resistant genotype, Auburn 56, to obtain the selection Auburn 834, which was more agronomically desirable than Auburn 623. Shepherd and colleagues then backcrossed Auburn 834 with five contemporary cultivars, selecting for RKN resistance

at each backcross, and made nine germplasm releases of final selections from these crosses (the M-series releases) (Shepherd et al. 1996). Pathology studies suggest that the RKN resistance mechanism involves two genes that retard development of the parasite and development of the giant cell at ~6 and ~24 days post penetration, respectively (Creech et al., 1995 and Jenkins et al., 1995). Further evidence of a two-gene system was obtained from crosses with the M series lines (McPherson et al., 1995). A conservative assessment of the resistance would be that one gene is partially dominant, and the second is additive in effect.

RKN Resistance – Status of Commercial Development

No commercial cultivars have been developed using the Shepherd germplasm releases. However, the cultivars 'Paymaster (previously Hartz) 1560' (a mid-season cultivar that is no longer commercially sold) and 'Stoneville (STV) LA 887' (a long season cultivar that is the backcross parent of 'STV 5599') were developed by Jack Jones at LSU from an elite breeding line RN-434 with a moderate level of RKN resistance inherited from Clevewilt 6 (one of the parents of Auburn 623). They are thought to carry one of two genes for RKN resistance present in Auburn 623. RKN resistance was also developed independently and apparently from entirely different sources in Acala cotton by Angus Hyer of the USDA-ARS working in collaboration with the CPCSD and the University of California at Shafter, California. This accomplishment involved approximately 20 years of screening phenotypes in the field. The resulting RKN-resistant Acala cultivar, 'NemX' is highly effective against RKN populations in California, but commercial adoption has been limited, because although NemX yields higher than other Acala cotton cultivars on nematode infested soil in California, it does not yield as high as other cultivars when nematodes are absent. Recent information strongly suggests that in 'NemX' the resistance is attributable to double-recessive inheritance (Roberts and Wang, reported herein). Commercial planting seed companies have infrequently used RKN resistant germplasm because selection must be done phenotypically on highly infested sites, a long-term process; or by using some screening method that depends on digging roots to score for galling or by counting nematodes. Representatives of the planting seed industry who were present stated that they needed: 1. HPR germplasm sources, 2. knowledge of the inheritance of HPR in the germplasm, 3. rapid PCR-based DNA assays for marker assisted selection (MAS) for nematode HPR. Development of MAS for this trait depends on discovery of closely linked flanking polymorphic DNA markers, which can be rapidly screened on plant tissue irrespective of environment.

RKN Resistance – Mapping Populations and Progress

Three segregating plant populations that were developed by separate projects in Georgia, Texas and California, are being investigated using bulk segregation analysis (BSA) and restriction fragment length polymorphisms (RFLP) (GA, TX) or amplified fragment length polymorphism (AFLP) (CA). In all of these cases, populations are from crosses of a *G. hirsutum* RKN resistance source and a susceptible *G. barbadense* or *G. hirsutum* genotype. Resistance in the Georgia population, 'M-120' x *G. barbadense* 'Pima S-6', is being mapped utilizing 200 RFLP markers provided by Andy Patterson. Similarly, the Texas population, 'M 315' x *G. barbadense* 'TX 110', is being compared with about 600 RFLP markers provided by Andy Patterson. In the Texas population, the two resistance genes have been roughly located. One gene is tentatively located on

chromosome 14, approximately 22 cM from pAR815 (Patterson laboratory nomenclature) and the other is on linkage group A02, 19 cM from B1-3 (Patterson laboratory nomenclature). In California, about 50 AFLP polymorphisms have been identified in recombinant inbred populations of 'Nem X' x *G. hirsutum* 'Acala SJ-2' and of 'Nem X' x *G. barbadense* 'Pima S-7'. Of these polymorphisms, about 19 appear promising for identifying resistant gene loci. In a different project in Texas, six out of 700 PCR primers tested have been found to provide markers uniquely associated with all five resistant members of five pairs of resistant and non-resistant sister lines developed previously by Shepherd by backcrossing resistance from Auburn 623 into five cultivars. To enable high-resolution mapping of the genes, this project has generated 25,000 seed of three populations (Auburn 623 X *G. hirsutum* 'Deltapine 16', Wild Mexican X Deltapine 16, and *G. barbadense* 'Pima S6' X Auburn 623) as well as F4 recombinant inbred lines from 202 F2s of the latter cross, and has confirmed the presence of the putative markers in 20 of 20 resistant F2's tested from this cross. A candidate gene approach is also being used in this project to fine-map and clone the genes. Furthermore, a BAC library for Auburn 623 and genome-wide physical map contigs for TM-1 have been constructed and will be used to targeted-develop multiple and readily usable DNA markers for the genes for commercial, high RKN resistant variety development in different cotton breeding programs.

Reniform Nematode – Resistance Genes from *G. hirsutum, G. barbadense*

Whereas resistance to reniform nematode is known in *G. barbadense* and certain primitive cotton relatives, no strong resistance is known in *G. hirsutum*. Based on the identification of accessions with some resistance to reniform nematode from two-tiered screening of the U.S. germplasm collection, Forest Robinson is undertaking a cooperative effort with Johnie Jenkins' laboratory. The two USDA-ARS laboratories will initiate a transgressive segregation breeding program intended to develop high levels of reniform resistance in *G. hirsutum*. The approach is similar to that previously done by Ray Shepherd for RKN resistance. In a program employing a replicated greenhouse screen followed by field confirmation, David Weaver is evaluating the available *G. hirsutum* material, and will identify and use the most resistant lines to initiate a program to breed for reniform nematode resistance at Auburn University. Jim Starr and Wayne Smith are developing a population from a *G. hirsutum* parent with RKN resistance and a *G. barbadense* parent with moderate reniform nematode resistance.

Reniform Nematode – Resistance from *G. longicalyx*

The wild, cotton relative, *G. longicalyx* has virtual immunity to reniform nematode. *G. longicalyx* is a diploid and thus not cross-fertile with *G. hirsutum.* Therefore, Al Bell constructed two triple hybrids: HLA = (*G. hirsutum* x *G. longicalyx*)^2 x *G. armourianum* and HHL = (*G. hirsutum* x *G. herbaceum*)^2 x *G. longicalyx*. A project has been initiated to develop backcross families using the triple hybrids and *G. hirsutum.* The backcross families will be evaluated for fertility, nematode resistance, and cytology by Al Bell, Forest Robinson, and Nilesh Dighe (grad student) and David Stelly, respectively. As nematode resistant lines are developed, David Stelly will pursue a concurrent program to map the resistance genes using BSA and AFLP. Preliminary work suggests that the resistance factors are on a single chromosome.

Research Progress and Plans

Root-Knot Nematode Resistance: Progress at the Starr and Chee laboratories suggests that rough location of the RKN genes may be anticipated by the end of project year 2004. Additional work will be needed to locate the genes within 1-0.5 cM, identify the genes themselves or suitable markers for MAS.

Reniform Nematode Resistance: The joint Bell-Robinson-Stelly project to exploit the *G. longicalyx* resistance source includes all the technical expertise necessary to produce cytologically described germplasm with mapped resistance genes. If resistance from *G. longicalyx* is conferred by a single gene, it may be possible to develop germplasm for release with a three-year scenario, i.e., project year 2006. Depending on the nature of the gene system and the rate of research progress, there is a good possibility that AFLP markers will be developed concurrently with the germplasm. At an appropriate point, PCR marker development should be initiated such that a complete package may be transferred to the plant breeding community at one time.

Development of Root-Knot Nematode (RKN) Resistance

Johnie Jenkins, USDA-ARS, Starkville, MS

Inheritance and Mechanism of RKN Resistance, and Current Efforts to Develop Advanced Lines with RKN Resistance

Fifty-six primitive and day-neutral accessions have been reported to exhibit some level of resistance to RKN. Thus, genes for RKN resistance are not uncommon in *Gossypium* species. At Auburn University, Ray Shepherd initiated a breeding program to develop a high level of RKN resistance in cotton starting with wild *Gossypium barbadense*. Transfer of resistance from *G. barbadense* into agronomic *G. hirsutum* types was unsuccessful. Shepherd succeeded subsequently, however, in developing a very high level of RKN resistance within *G. hirsutum* through transgressive segregation, by crossing a primitive accession (Mexican Wild = TX 2516) with a moderately resistant contemporary cultivar, 'Clevewilt 6-8' (Shepherd, 1974a) and making repeated selections for resistance in filial lines. Ultimately, 'Auburn 623' was released from this program and became a resistance source (Shepherd et al. 1974b). Nine resistant lines were developed and released by backcrossing resistance from 'Auburn 623' into then contemporary cultivars (the "M series lines"), and seed of each was deposited in the National Seed Storage System (Shepherd et al., 1996). The inheritance of RKN resistance in the M series has been described (McPherson et al. 1995). The histology of the RKN resistance mechanism has been described in 'M315' (Creech et al, 1995). Six to eight days after inoculation the number of nematodes declines, then declines again after 24 days. In 'M 315,' there is evidence for a dominant gene from Mexican Wild and a recessive gene from 'Clevewilt 6-8'. The USDA-ARS laboratory at Starkville is continuing to investigate the mechanism at the molecular level, and continuing to make crosses with modern cultivars to produce advanced breeding lines. Evaluations of RKN resistance in current commercial cultivars indicate that the cultivars reported to be RKN-resistant do not carry all the resistance genes. Several RKN resistant lines are in advanced stages of evaluation in a research program and in Mississippi State tests. Several RKN resistant lines have produced yields comparable to those of 'SG 501', 'SG 747' and 'STV 474'.

Peng Chee, Lloyd May, University of Georgia and Richard Davis, USDA-ARS Tifton, Georgia

Inheritance of RKN Resistance and Progress in Marker Development

In order to develop markers for RKN resistance-genes, a population was synthesized from crossing *G. hirsutum*, 'M 120' and Pima S-6, *G. barbadense*. Richard Davis screened the F_1 by rating galls, enumerating total eggs, and calculating eggs/gram of root. The F_1s were highly resistant to RKN. F_1s were self pollinated to create 226 F_2 progenies. Two hundred RFLP markers have been made available by Andy Patterson. The group of markers have been selected to be about 25-30 centimorgans (cM) apart to cover the entire genome. About one third of the work has been done. Other work has demonstrated that there is a general lack of polymorphisms for these markers in cotton. Therefore, the effort will likely locate the genes at a very rough scale, and the current RFLPs will not be useful in MAS. Therefore, additional fine mapping will be necessary. In addition to this effort, back cross breeding is being done with 'M-120' and selected lines. Richard Davis is doing the resistance evaluations. At BC_3, the lines should no longer be segregating and elite resistant lines will be released. Research plans are to be in the field next summer evaluating lines for agronomic characters.

Jim Starr, Texas A&M University, College Station, Texas

Inheritance of Resistance to RKN

A population has been developed by inter-specific crossing of *G. hirsutum*, 'M 315' and TX 110, *G. barbadense*. 'M 315' has RKN resistance from 'Auburn 623', and 'TX-110' has reniform nematode resistance. Bulk segregation analysis is being used to screen for RFLP loci. A total of 192 of 566 RFLP markers have been screened. Probe pAR815 is located on chromosome 14 (C14) at 21.6 centimorgans from a resistance locus. Probe B1-3 maps at 18.6 cM from a second resistant gene on linkage group A02. The intention is to follow up and achieve closer identification of the gene loci by using additional RFLPs and SSRs. Roy Cantrell indicated that such SSRs could be found in Lacape et al. 2003 for these two chromosome/linkage groups.

Hongbin Zhang, Texas A&M University, College Station, Texas

Genomic Tools for RKN Resistance Breeding in Cotton

A program was initiated in cooperation with Forest Robinson, Charlie Cook, John Yu, and Russ Kohel to develop mapping populations and identify RKN resistance genes. Initial examination of 700 oligo-PCR primers (RAPDs) identified six that provided DNA bands uniquely associated with resistant sister lines among five near isogenic lines generated by Shepherd by backcrossing resistance from Auburn 834 into five commercial cultivars. To enable high-resolution mapping of the genes, some 25,000 seeds of three populations were generated from crosses of *G. hirsutum* x *G. hirsutum* (Auburn 623 x Deltapine 16 and Mexican Wild x Deltapine 16) and *G. hirsutum* x *G. barbadense* (Auburn 623 x Pima S-6). Segregation of resistance in all cases suggested two major genes. The presence of the markers identified in the resistant isolines was confirmed in 20 of 20 resistant F_2 tested from the interspecific cross. From the F_2 generation of the interspecific cross, recombinant inbred lines (RILs) are being

generated to reduce assay-dependent variance in phenotype assignment and RILs are now at the F_4 generation because phenotyping is the key to accurately mapping and successfully developing reliable DNA markers for the genes. A candidate gene approach based on analyses of the disease resistance gene analogs was also used. Disease resistance gene analogs were cloned from Auburn 623, an arrayed library was constructed, a large number of the clones were sequenced and are being used to map and clone the RKN resistance genes. Furthermore, to high-resolution map and clone the genes, this group has constructed a large-insert BAC library for Auburn 623 and generated contigs from approx. 100,000 (5.6x) BACs and BIBACs of the Upland cotton genetic standard TM-1. The Auburn 623 BAC library and the TM-1 contigs will be used to develop multiple DNA markers that are suited for commercial, highly RKN resistant cotton cultivar development in different cotton breeding programs.

Phil Roberts and Congli Wang, Univ. of California at Riverside

Genetic and Molecular Characterization of Host-Plant Resistance to RKN and Fusarium Wilt in Cotton

The project objectives are to determine the uniqueness of the genetic basis of resistance to RKN and Fusarium wilt in 'NemX', develop molecular markers linked to the RKN and Fusarium resistance genes (R), localize the R-genes on chromosomes, and develop high resolution linkage maps around the R-genes. The parents, 'SJ-2', 'NemX, and 'Pima S-7' are susceptible, resistant, and susceptible to RKN, respectively. RILs have been developed and will be carried forward to the F_7 or F_8 generation. The $F_{2\text{-}3}$ data suggests that RKN resistance in 'NemX' is associated with two recessive genes. Marker development is progressing using BSA with AFLPs. Fifty polymorphisms have been identified, of these 19 seem promising for identification of gene loci. Future work includes screening the markers for linkage to the RKN and Fusarium R-genes and confirming the gene interaction that determines the resistant phenotypes. The intention is to convert the markers to STS/SCARs, localizing the genes to chromosomes, and achieving fine mapping of the resistance genes.

Molecular Approach to Development of Novel Means of Nematode Control

Andrew Kloek, Divergence, Inc.

Developing Safe, Effective Products for the Control of Plant Parasites

Divergence is a Research and Development company that is looking for genome-based approaches to discover novel nematicides and nematode-resistant plants. Identifying safe targets is difficult, since humans and nematodes share approximately one half their genes, and essential genes tend to be conserved. However, public genomic information is expanding rapidly. There are more than 200,000 published expressed sequence tags (ESTs) and more than 100 cDNA libraries that depict genetic information on 26 species of nematodes. Divergence's approach is to use bioinformatics to screen the genomic data and target genes for inhibition using RNA interference. The targets are chosen based on their specificity in the nematode and absence in mammalian genomes. Successful gene inhibitors may become candidate nematicides, plant genes,

anti-parasitics, or vaccines. Target genes fall into distinct classes: 1- enzymes of known function, 2- enzymes whose function remains unknown, 3- secreted or trans-membrane proteins (chiefly in the nematode intestine), and 4- nematode-specific proteins of unknown function. Divergence is concentrating on approaches 1 and 3. In addition, two approaches have been used to make transgenic plants that are resistant to nematodes. The approaches are engineering plants to express small-molecules that inhibit nematode-specific biochemical pathways, or small polypeptides that inhibit essential proteins in the nematode intestine. Divergence has successfully identified two small-molecule inhibitors, and demonstrated the efficacy of the concept by inhibiting biosynthesis and restoring function in *C. elegans* by administering high doses of the inhibited product.

Development of Reniform Nematode Resistance

Forest Robinson, USDA-ARS College Station, Texas and cooperators

Resistance to Reniform Nematode in four Gossypium species masked by Nematode Survival in Texas, Louisiana, Mississippi, and Alabama

(Presentation at Society of Nematology Annual Meeting, July 13, Ithaca, NY)

Nematode resistance is typically evaluated in pots. In a 2001 test in South Texas, four *Gossypium* species, previously identified as resistant in greenhouse evaluations, showed little capacity to reduce reniform nematode populations in the field. The experiment was repeated in 2002 at four locations. At each location, root mass and nematode populations were measured at harvest in 15-cm horizons to the 120-cm depth. Cotton root and nematode distribution varied widely among locations, indicating that characterization of cotton rooting at each location is necessary, before relevant sampling of reniform nematodes can be done. Analysis of gross reniform nematode numbers suggested that again the resistant accessions had not suppressed nematode numbers. However, reniform populations were also enumerated in fallow areas, where no plants were present. Populations in these areas were 30-40% of those in the proximity of cotton plants. When the populations in the fallow areas were subtracted from those in the soil sampled from around the roots of the cotton accessions, the analysis of the adjusted populations indicated that the resistant accessions were associated with lower populations than were susceptible accessions. These data show that large numbers of reniform nematodes may be found in fields that have previously grown susceptible crops, and assessments of resistance and susceptibility of genotypes must also consider patterns of root development and overall nematode populations.

Forest Robinson, USDA-ARS, College Station, Texas

Evaluation of Primitive Accession of *Gossypium hirsutum* and *G. barbadense* in the National Cotton Collection

A two-stage screen was used to evaluate 2093 and 1197 *G. hirsutum* and *G. barbadense* accessions, respectively. In the first level screen, plants are grown in pots for 10 weeks. From one plant of each accession, the at-harvest number of nematodes was compared with two standards, the numbers of nematodes on susceptible ‘DPL 16’

and on the weakly resistant 'TX 1348'. Accessions with fewer nematodes than 'TX 1348' were classified as possibly resistant and evaluated again in a replicated test that was conducted in a growth chamber to reduce variability due to plant growth. In general, resistant plants always had some nematodes present, but susceptible plants never had low numbers, although numbers of nematodes tended to be highly variable between susceptible plants. Ratings were based on reproduction relative to 'DPL 16'. Those accessions with mean numbers of nematodes less than 2/3 but more than 1/3 of 'DPL 16' were rated as weakly resistant, while those with less than 1/3, but more than 1/10, or less than 1/10 as many nematodes per gram of root of 'DPL 16' were rated moderately resistant and resistant, respectively. Among candidate *G. hirsutum* accessions that were retested, 12 and 6 were rated weakly and moderately resistant, respectively. One accession, 'TX 502' was highly resistant, but it appeared to be *G. barbadense* based on the appearance of the phenotype. Among candidate *G. barbadense* accessions that were retested, 8, 19, and 4 accessions were classified as weakly or moderately resistant, or resistant, respectively.

David Weaver and Kathy McLean, Auburn University

Screening the *G. hirsutum* collection for resistance to *R. reniformis*

The long-term objective is to evaluate all available accessions of *G. hirsutum* for reaction to the reniform nematode, to determine the iinheritance of resistance if detected, and to incorporate resistance into improved lines. Accessions first are evaluated in the greenhouse using four replications of single plants that are inoculated with a mix of reniform nematode populations from different geographic areas (1000 vermiform/150 cc of soil). After 60 days, populations are determined and the reproduction factor calculated. Vermiforms are an indication of survival, and eggs are an indication of reproduction. Accessions in the lowest 10 percentile for each parameter will be advanced to the next level. Final evaluation is done in the field. Approximately 1000 accessions or 25% of the total have been evaluated. Paymaster 1218 is used as a standard in every set of 50 accessions that are done at one time. Analyses of log transformed vermiform and egg data suggest that there would be some differences in the accessions selected if either the vermiform or the egg data were used alone. Therefore, accessions are selected when they are in the lowest 10^{th} percentile in both vermiforms and egg counts. Of the 865 accessions that have complete data to this point, four accessions will be planted in the greenhouse to begin a crossing program.

Jim Starr, Texas A&M University

Inheritance of Resistance to Reniform Nematodes

Resistance to reniform nematode in the *G. barbadense* line, 'TX 110' was reported (Yik and Birchfield, 1984) and confirmed by F. Robinson and Starr. Prior to the current work, there was no data on the inheritance of the resistance. A population was made by crossing 'M315', from the RKN-resistant Shepherd material, with 'TX-110'. Resistance to RKN was confirmed in the F_1, but resistance-susceptibility to reniform nematode in the F_2 exhibits a distribution characteristic of a quantitative trait, suggesting that reaction to reniform nematode was conditioned by multiple genes (possibly five, as suggested by C. Gill, personal communication) with one of the resistance genes being partially

dominant. The research plan is to screen for RKN and reniform nematode in alternate generations. Presently the $F_{3:4}$ is being selected for RKN resistance, and the $F_{4:5}$ is being selected for reniform nematode resistance. Bulk segregation analysis cannot be used effectively for a quantitative trait. Of 192 RFLP probes that have been tried, no linked markers have been identified.

David Stelly, Nilesh Dighe, Texas A&M University and
Al Bell and Forest Robinson, USDA-ARS, College Station, Texas

Introgression of reniform nematode immunity from *Gossypium longicalyx* into Upland cotton (*G. hirsutum*): Cytology, Genetics, and Gene Tagging

The cotton relative, *G. longicalyx,* has virtual immunity to reniform nematode, but it is a diploid species that is reproductively incompatible with *G. hirsutum*. Al Bell has made two types of triple hybrids: HLA = (*G. hirsutum* x *G. longicalyx*)^2 x *G. armourianum* and HHL = (*G. hirsutum* x *G. herbaceum*)^2 x *G. longicalyx*. The objective is to transfer the immunity to reniform nematode to *G. hirsutum,* determine the genetic control and inheritance of the trait, and create resources that will expedite breeding of immune cultivars. Backcross populations will be made and evaluated for fertility and nematode susceptibility by Al Bell, and Forest Robinson. Nilesh Dighe and David Stelly will evaluate the cytology and develop relevant markers. At present BC_1 and BC_2 populations of the triple hybrid sources and *G. hirsutum* have been made and are being evaluated. Of 80 plants that have been evaluated, 64 have a full complement of 52 chromosomes. Evidence of recombination has been observed, and observations suggest that the immunity factor or factors may reside on one chromosome. (However, the latter observation must be supported by additional data.) The team expects to develop molecular markers closely linked to the immunity gene as an on-going part of the project using a BSA – AFLP approach. Upon introgression, user-friendly markers will be developed for MAS.

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