

TOWARDS STUNT-FREE LONREN RENIFORM NEMATODE RESISTANCE BY
DISSECTION OF AN ALIEN R-GENE CHROMOSOME SEGMENT AND
DETECTION OF SIGNIFICANT FUNGAL INTERACTIONS

A Dissertation

by

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ABSTRACT

Reniform (*Rotylenchulus reniformis*) nematodes cost US cotton growers over 130 million dollars per year. Genetic resistance of the wild African species *Gossypium longicalyx* to reniform nematodes was previously transferred into upland cotton (*Gossypium hirsutum* L.). LONREN and other cotton lines containing the responsible gene, Ren^{lon} , are highly nematode-resistant, but when grown in nematode-infested fields, the seedlings and plants became "stunted". Hypothesized causes include hypersensitivity reaction, *per se*, and/or increased susceptibility to soil pathogens. My research objectives have been: [1] to develop Ren^{lon} -linked markers and map the Ren^{lon} region at high resolution, [2] to recombine Ren^{lon} and nearby loci and determine if "stunting" is due to linkage drag, [3] to create genomic and germplasm resources for genetic manipulation of chromosome-11, especially Ren^{lon} , and [4] to determine if "stunting" is due solely to plant-nematode interactions or involves additional pathogens.

Putative SNPs between *G. hirsutum* and *G. longicalyx* were collaboratively sequence-aligned to the D5 reference genome. By selecting SNPs aligned to the Ren^{lon} -homeologous region and screening them against a genetic panel, we identified 85 as Ren^{lon} -linked. To enable large-scale SNP applications, we developed inexpensive methods for high-throughput non-destructive seed DNA extraction for PCR-based genotyping. We then high-resolution mapped 10 SNPs in the proximal alien segment near Ren^{lon} . We chose the two closest Ren^{lon} -flanking SNPs and used them for marker-

assisted selection to identify 5 recombinants from 17,600 BC1F1 seed. Subsequent progeny tests indicated 2 of the 5 recombinants were free of "stunting" and that 1 seems to be segregating for nematode resistance. Contingent on verification of the resistance, the results indicate that stunting of LONREN seedlings is indeed due to linkage drag, not hypersensitivity, *per se*. To determine if "stunting" can be ascribed solely to hypersensitivity-induced root damage, we conducted three replicated growth chamber experiments with multiple genotypes, pasteurized and natural soils, and several pathogen treatments. The results showed that *Thielaviopsis* root rot alone can cause severe seedling "stunting", but not the nematodes, alone. Moreover, they showed that LONREN-like genotypes are differentially sensitive to the combined presence of reniform nematodes and *Thielaviopsis*, and become more severely stunted.

DEDICATION

A special feeling of gratitude to my loving parents, Xiang Zheng and Xiaoxia Wang, to my husband, Ming Zhang.

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CHAPTER I

INTRODUCTION

Cotton and Impact of Reniform Nematode

Cotton is the leading natural fiber crop and is produced by plants of several species of the genus *Gossypium* L., which includes extensive phenotypic diversity among *ca.* 52 species, including the two new species recently identified (30) (Grover et al. unpublished, Wendel et al. unpublished). The genus consists of at least five allotetraploid ($2n = 4x = 52$) and 45 diploid ($2n = 2x = 26$) species (56). There are four domesticated species, including the New World allotetraploids *G. hirsutum* and *G. barbadense* and the Old World diploids *G. arboreum* and *G. herbaceum*. *Gossypium hirsutum* and *G. barbadense* originated in the Yucatan Peninsula of Central America (145, 163). Other species of cotton serve primarily as germplasm sources for genetic improvement. Of the four domesticated cottons, *G. hirsutum* is the most important domestically and worldwide. While best known for the fiber that it produces on the surface of seeds, the cotton seed is also a valuable source of vegetable oil, ruminant animal feed and other feed products (42).

The primary species of cotton grown in the United States is *G. hirsutum*, also called upland cotton, although Pima cottons are also grown in certain regions, and are primarily *G. barbadense*, infused with small amounts of *G. hirsutum* germplasm through hybridization and human selection. Pima cultivars are characterized by extremely long

fibers (>4 cm), but are grown on limited hectares in the western states of New Mexico, Arizona, and California, and accounts for less than 5% of the total U.S. crop.

Cotton is a perennial plant that is generally grown as an annual in cotton production systems. The United States was ranked third in lint production for the year 2010, after China and India (USDA-Foreign Agriculture Service). The amount of US land devoted to cotton production peaked in 1926 at approximately 18 million hectares. The advent of mechanized farming and the availability of effective, relatively low-cost fertilizers, pesticides and improved cotton cultivars after World War II allowed for significantly greater yields per unit area, leading to a decline in cultivated hectares. High-yielding cultivated Upland cotton cultivars have been considered genetically vulnerable for many years (68). Molecular data underscore the low genetic variation at the species level, relative to other plant taxa (29), with further reductions resulting from domestication, selection in the USA for non-photoperiod sensitivity and predominant modern breeding practices. The reduced genetic variability increases potential vulnerability to pathogen or insect epidemics of the crop (25).

The importance of plant-parasitic nematodes as yield-limiting pathogens of cotton has received increased recognition and attention in the United States in the recent past (141). Currently, the four most damaging species of plant-parasitic nematodes affecting cotton in the US are the southern root-knot (*Meloidogyne incognita*), reniform (*Rotylenchulus reniformis*), Columbia lance (*Hoplolaimus columbus*) and sting (*Belonolaimus*

longicaudatus) nematodes (22, 23, 85, 92). Estimated losses of cotton lint yield by these pathogens in the US have increased from 1% to 2% in the 1950s to more than 4% in 2000 (22). Nematodes are the pathogens that cause the greatest losses in U.S. cotton, and reniform nematode is second only to root-knot nematode (*Meloidogyne spp.*), causing an estimated economic loss of approximately 2.0% nationwide. However, in the Mid-South states of Alabama, Mississippi, and Louisiana, losses to reniform nematode have been much higher, averaging 7.2% (24). Some areas of Texas also suffer significant losses.

Reniform nematode was first reported as a pest of cotton in 1940 by A.L. Smith (1940). Originally described in Hawaii (90), reniform nematode was found associated with *Pratylenchus pratensis* (probably *P. brachyurus*) in Georgia fields used in fusarium wilt trials by Steiner and Smith (138). Subsequently, reniform nematode was identified as the causal agent for stunted cotton in Louisiana in the 1960s (18, 19, 73). Important studies on reniform nematode were conducted in Louisiana and Texas in the 1970s and 1980s, but only in the past decade has there been an increase in research effort and heightened awareness of the pathogenic ability of this nematode.

Unlike many other species of plant-parasitic nematode, reniform nematode reproduces readily and achieves very high population densities in fine-textured soils. The life cycle of reniform nematode follows the basic pattern for plant parasitic nematodes, beginning with a one-celled egg. The egg is deposited in a gelatinous matrix and undergoes cell divisions that lead to the development of the first-stage juvenile (J1). The J1 molts to

form a second-stage juvenile (J2) that hatches from the egg. The life cycle of the juvenile stages diverges from that of many plant parasitic nematodes in that the J2 through J4 stages are fairly immobile and do not feed (20, 58, 127, 137). Also, the J3 and J4 remain inside the cuticles from previous molts (20). Adult males and immature females emerge from the cuticles, and vermiform females penetrate roots to establish a feeding site in the stele. The females are sedentary semi-endoparasites during the remainder of their life cycle, with the anterior portion of the female on the root surface. The reproductive system of the female continues to develop after initiating a feeding site, and egg production begins within 1 to 2 weeks, depending on temperature. Typically, about 60 eggs are found in an egg mass. The optimum temperature for reproduction is about 30°C, with no development at 16 or 36°C (20).

Reniform nematode can feed and reproduce on a wide range of plants. Many vegetable crops, vetch, clover, and certain varieties of soybean are excellent hosts. Non-host crops include corn, sorghum, peanut, and winter grain crops such as wheat, rye, oats, and barley (59). Reniform nematode feeding induces marked cellular changes, including formation of dense granular cytoplasm, dissolution of cell walls and coalescing of cytoplasm of adjacent cells, thickening of cell walls by deposition of polysaccharides, enlargement (hypertrophy) of cells and enlargement of nuclei and nucleoli, resulting in the formation of multinucleate syncytia (118). Carter (31) reported that in reniform-resistant *G. arboreum*, feeding leads to hypertrophy of pericycle cells, and their disintegration starting at 7 to 12 days after penetration. Cell walls immediately adjacent

to the nematode head were thickened and more safranin-positive in resistant than in susceptible cotton cultivars (31).

Nematode Management in Cotton

Management by cultural practices

In modern cotton production, cultural practices often have limited use in suppressing nematode population densities and minimizing yield losses (120). Crop rotation, growing non-host, resistant, or antagonistic cover crops, incorporation of plant materials or animal manures, and destruction or removal of cotton stalks and roots to minimize nematode survival and reproduction have been investigated (7, 44, 45, 81, 83). Tillage has long been recommended as a means of incorporating crop residue and for destruction of residual roots. This may be especially important for cotton since it is a perennial and could support reproduction of plant-parasitic nematodes in areas where soil temperatures remain above the activity threshold for pathogenic nematodes for extended periods following harvest of the crop. Destruction of cotton root systems or removal with a stalk puller, however did not increase the yield of subsequent cotton crops in Georgia or North Carolina (35, 68). Additional cultural practices that have been suggested for suppressing nematode population densities include planting date, the use of organic amendments, and cover crops. Unfortunately, little information is available on the effects of planting dates on cotton nematode populations. Cotton planting date had no impact on Columbia lance nematode in North Carolina, and planting dates are not flexible in many areas because of the relatively long season used to produce cotton in the United States (81).

Management with nematicides

Nematode management in cotton is largely dependent on nematicides, and these materials are reasonably effective in preventing cotton-yield suppression by plant-parasitic nematodes on cotton (139). The use of nematicides, however, has increasingly come under scrutiny by public and government agencies because of toxicological and environmental concerns (149). Currently, the only nonfumigant nematicide/insecticide with a proven level of efficacy against nematodes that is labeled for at-planting use in cotton is aldicarb (Bayer Crop Science, Research Triangle Park, NC). Another carbamate, oxamyl (DuPont Crop Protection, Wilmington, DE), is labeled for foliar application to cotton, provided a preplant or at-plant application of a nematicide was made. Current fumigant nematicides labeled for use in cotton include the chlorinated hydrocarbon 1, 3-dichloropropene (Dow AgroSciences, Indianapolis, IN) and metam sodium (Amvac Chemical Corp., Los Angeles, CA).

Management by biological control

Over the past 20 years, studies on biological control of reniform nematode have reported antagonistic activity of nematophagous fungi and strains of bacteria against different life-cycle stages of nematode (156-158). *Paecilomyces lilacinus*, *Pochonia chlamydospora*, and an unidentified fungus named Arkansas Fungus have been documented as parasites of the egg stage of reniform nematode (77, 157). The vermiform life stages were reported to be colonized by the fungi *Arthrographis* spp., *Pseudorobillarda* spp., and *Fusarium equiseti* (101). The zoosporic fungus *Catenaria*

auxiliaris was reported colonizing reniform nematode vermiform life stages (32).

Furthermore, strains of the bacteria *Pasteuria* spp. and *Pseudomonas fluorescens* have been reported to reduce the number of reniform nematodes in soil (65, 133). Recently, soils suppressive to reniform nematode have been reported in Louisiana and Texas; however, the agents responsible for this suppression have not been identified (128).

In summary, chemical control is somewhat successful, but it is expensive and environmentally damaging and only a temporary solution. Crop rotation, whenever possible, is a better management alternative (26, 45, 144, 148, 164). But, rotation often returns only a fraction of the profits lost to reniform nematode damage. Most rotational crops are less profitable than cotton and the performance boosts to subsequent cotton is limited to only the first year that cotton is grown following the rotation (45). Biological control holds some promise for the future, but with current knowledge it is difficult to promote or establish a microflora or fauna in soils that effectively suppresses nematode population densities, especially in the relatively short period of time of a single growing season. Reliable and effective biological control systems are likely to be limited in the near future to specialized situations, such as intensely managed crop systems where the environment can be manipulated to promote biological activity. Ultimately, therefore, host plant resistance is the best means of reniform nematode control from economic perspectives, as well as from environmental and human health perspectives. Host plant resistance has been prioritized over chemical, biological, cultural, and regulatory control components as a major goal for pest management (139). The desirability of genetic

resistance as a solution to the reniform nematode has increased with growing awareness of real and perceived dangers from some nematicides, and as laws have increasingly restricted their use (41, 52, 55, 121, 165). If developed by conventional hybridization methods using resistance genes that occur naturally in *Gossypium*, these products would not be transgenic, and could be readily deployed in US grower fields. Another, partial solution has been to identify tolerant genotypes that suffer less damage than do typical cultivars. A drawback of tolerance is that tolerant genotypes typically support high levels of nematode reproduction, and thus cannot be used to reduce the nematode population density in the soil. A second drawback is that tolerance appears to be highly environment-dependent, making the development of widely adapted tolerant cultivars unlikely (82). More than a dozen breeding lines and cultivars exhibiting some degree of reniform nematode tolerance have been identified (37-40, 72, 82, 134, 143, 150). Because discovery of tolerance provides an immediate solution to the problem, the search for tolerance remains an important research priority. Recent studies have identified several breeding lines and cultivars with potential for use in the important Mississippi Delta production region (134, 143).

Interaction Between Soil Pathogens and Reniform Nematode

Interactions between nematodes and other plant pathogens have been documented in a number of crops (1, 2, 33, 53, 140, 142, 155, 161). Synergism between nematodes and fungal pathogens in cotton is much more common, or perhaps better documented, than associations between nematodes and other pathogen groups (142). In much of the U.S.

Cotton Belt, interactions between nematodes and seedling disease pathogens are common. In the early 1950s, an increase in cotton seedling disease, caused by *Rhizoctonia solani*, was observed in the presence of *Meloidogyne incognita* (119). This association with *R. solani* is not limited to *M. incognita*. Studies have suggested that increasing populations of reniform nematode and *R. solani* result in a linear suppression in plant growth (132). The presence of *M. incognita*, reniform nematode, or *Hoplolaimus spp.* has also been shown to increase the severity of seedling disease caused by certain fungal pathogens including *Pythium*, *Alternaria*, *Glomerella*, and *Fusarium spp.* (142).

Recently, numerous researchers have reported on interactions between *T. basicola* and *M. incognita* (71, 94, 104, 154, 155). It also has been reported that reniform nematode and several fungal species, including *T. basicola* are synergistically deleterious (108). With these interactions, enhanced seedling mortality and delayed plant development occur with cotton seedlings in the presence of both organisms. It seems that while each of these pathogens normally can cause disease of a chronic nature, in combination they can cause significant plant mortality, and there is some evidence that infection by the nematode allows *Thielaviopsis. basicola* access to vascular tissue that would not normally be invaded in the absence of the nematode (153).

Stunted plants of LONREN show symptoms typical of fungal root rots. A wide array of fungal pathogens, including *T. basicola*, have been isolated from the roots of nematode-

infested plants (16). The soil-borne fungus, *Thielaviopsis basicola* (Berk. & Broome) Ferraris (syn. *Chalara elegans* Nag Raj & Kendrick), the causal agent of black root rot of cotton, is an important plant pathogen of upland cotton (*Gossypium hirsutum* L.). It was first described in a field in Arizona in 1942 (78).

Currently, *T. basicola* is recognized as an economically important seedling pathogen of cotton throughout the world (5, 66). The fungus has a host range of over 230 species and is a destructive pathogen on the roots of many crop and ornamental plants (131). It overwinters as thick-walled chlamydospores that germinate in the presence of the host and adequate soil water (67). *T. basicola* usually colonizes the root cortical tissue, causing dark brown to black discoloration of roots and hypocotyl (97, 99). Black root rot is most severe when soils are cool (below 24 °C) and wet (above -20 joules/kg) (130, 153). Infection of the seedling affects the roots and the portion of the hypocotyl below soil level. Infected cortical tissue turns black, but the vascular tissue, which is often not attacked, remains white. Diseased seedlings are stunted and easily pulled from the soil. Upland cultivars are rarely killed by the disease if soil temperatures are above 20 °C, but affected plants may still be stunted, with a pronounced swelling in the crown area just below soil level (21).

Management approaches for most fungal–nematode disease interactions require a consideration of both pathogens. The use of nematicides has been shown to lower the amount of crop loss caused by both fungal and nematode pathogens (36, 74). Seedling-

disease pathogens, though, are capable of causing disease in the absence of the plant-parasitic nematodes, and the use of fungicides may also be necessary. Traditional disease management programs that utilize host plant resistance, crop rotation, and/or modification of the planting environment to enhance seedling vigor are also effective at limiting the damage caused by nematode–fungal interactions.

Cotton Resistance Breeding and Genomics

In an extensive survey of *Gossypium* germplasm for reniform nematode resistance and tolerance, Yik and Birchfield found *G. longicalyx* J.B. Hutch. & B.J.S. Lee, to be immune to reniform nematode infection, whereas *G. somalense* (Gürke) J.B. Hutch. and *G. stocksii* Mast. were highly resistant. Resistance was also found in *G. arboreum* L., *G. herbaceum* L. and *G. thurberi* Tod. accessions (170). The most information regarding resistance to the reniform nematode introgression is available for *G. barbadense*, *G. arboreum*, *G. aridum* and *G. longicalyx*. Most accessions of *G. barbadense*, a species which freely hybridizes with *G. hirsutum*, are susceptible to the nematode, and resistant *G. barbadense* accessions usually suppress nematode populations by only 70-90% (125). In contrast, many accessions of *G. arboreum*, from which genes are introgressed via bridging species, are highly resistant to the reniform nematode (146), and the most resistant *G. arboreum* accessions suppress nematode reproduction 95% or more compared to susceptible *G. hirsutum*. As the extreme case, *G. longicalyx*, from which genes can be transferred only with great difficulty, is virtually immune. Given that strongest reniform nematode resistance has been found among diploid species, wide-

cross introgression of the resistance gene(s) into upland and Pima cultivars is a logical step, although not an easy one. This apparent inverse relationship between compatibility and resistance within *Gossypium* greatly confounds strategies and funding for developing resistant cultivars.

The first successful genetic introgression into upland cotton of strong reniform nematode resistance from an alien species involved germplasm from the African diploid wild species *G. longicalyx*; it involved the synthesis and backcrossing of di-species hexaploid, a tri-species hybrid, "HLA", [$(G. hirsutum \times G. longicalyx)^2$] $\times G. armourianum$ Kearn.], followed by modified backcrossing (13, 124). Segregation of the reniform nematode resistance suggested monogenic control, and linkage with molecular and phenotypic markers indicated a single dominant gene or haplotype in chromosome 11, Ren^{lon} (48). Two of the resulting germplasm lines from this resistance source were jointly released by the USDA, Texas AgriLife Research and Cotton Incorporated, namely LONREN-1 and LONREN-2 (14).

Since the development and informal release of LONREN-1 and LONREN-2, progeny derived from a cross between a tri-species hybrid [$G. arboreum \times (G. hirsutum \times G. aridum)^2$] and MD51ne (*G. hirsutum*) showed that resistance among introgression products was associated with the SSR markers BNL3279_132 and BNL2662_090 of chromosome 21 and originally designated the alien c21 locus as Ren^{ari} , which is from *G. aridum* (129). To avoid confusion with the Ren^{lon} gene from *G. longicalyx* located on

chromosome-11, which nomenclature convention implies as an allele of the *Ren₁* locus, the name of the *Ren^{ari}* locus was later revised to *Ren₂^{ari}* (54). Current data also suggest that *Ren₁* and *Ren₂* loci are duplicate (homeologous) genes with *Ren₁* residing on chromosome 11 (member of the "A" sub-genome) and *Ren₂* on chromosome 21 (member of the "A" sub-genome). Plants containing both *Ren₁^{lon}* and *Ren₂^{ari}* appeared to have slightly higher resistance than those with just one gene. Pyramiding these two genes could also increase “durability” of the resistance.

Gossypium barbadense GB-713 is the most resistant of *G. barbadense* and *G. hirsutum* accessions noted to date (123). Robinson initiated attempts to combine resistance to reniform nematode with resistance to root-knot nematode (*M. incognita*) by crossing GB-713 with either Acala NemX or M 315-RNR. The germplasm line BARBREN 713 was released early 2012. A bulk-segregant analysis indicated the resistance to reniform nematode in was controlled by a single dominant gene with additive effects (125). Based on segregation and linkage analyses from progenies involving hybrids with Acala Nem-X, resistance of GB-713 was attributed to three resistance genes, including two in chromosome 21, and one in chromosome 18 (61). The resulting germplasm line from this resistance source BARBREN 713 were joint released by the USDA, MAFES, Texas AgriLife Research and Cotton Incorporated.

The evidence above collectively indicates that the best approach is to develop reniform nematode resistance in Upland cotton simultaneously through introgression of

resistances from multiple known sources. To facilitate introgression and gene pyramiding, it is absolutely essential to develop molecular markers for each resistance gene, because these enable researchers and seed companies to more cost- and time-effectively monitor sexual transmission, recombination and homozygosity of resistance as they proceed through the cultivar development process. Without molecular markers, reniform nematode resistance of individual plants can be detected only by nematode reproduction assays, which are time- and resource-consuming, expensive and not without error (48, 124).

Simultaneous introgression of resistance from several species is probably the wisest approach. Incomplete expression or incorporation of closely linked deleterious genes is possible in all cases. Resistance from a single source may prove inconsistent across locations and/or time. Pyramided resistance genes may provide higher degrees or more durable resistance. The availability of more than one gene resistance might allow for varying the resistance to reduce likelihood of the pathogen developing virulence to a single resistance gene.

“Stunting” Problem and the Hypotheses

LONREN-1 and LONREN-2 were shared soon after their development and before official release to expedite performance evaluations in additional locations, experimentation and breeding. Broader evaluations of the LONREN lines and derived materials quickly revealed that plants carrying plants bearing the alien *Ren^{lon}*-bearing

segment were debilitated when grown in heavily nematode-infested fields. LONREN and derived resistant lines were smaller and less productive on average than isogenic susceptible lines in spite of their resistance to the nematodes(15). To avail growers the maximum benefits of the nematode resistance, cultivars with resistance should be free of special concerns and, if possible, not require specialized weed management practices. So, eliminating the "stunting", if possible, is deemed to be an important practical goal. There are six hypotheses for LONREN stunting (**Fig. 1.1**).

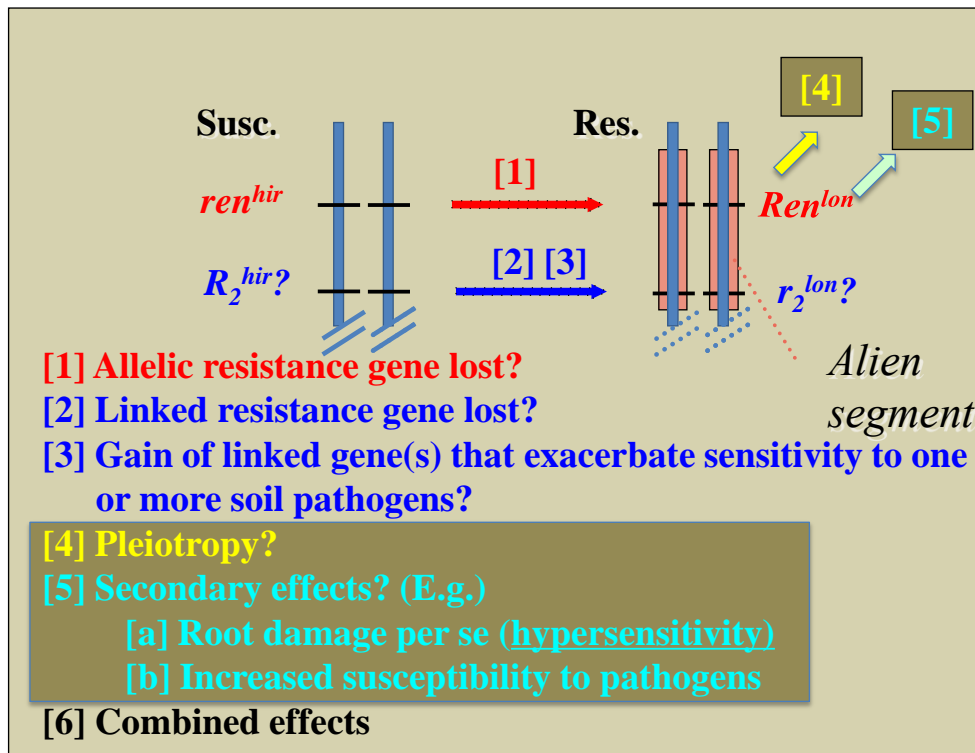


Figure 1.1. Primary hypotheses for stunting of LONREN lines in the field.

1. Allelic resistance gene loss: It is possible that *G. hirsutum* chromosome 11 (c11) normally contains an “allele” of the *Ren^{lon}* gene that contributes significantly toward plant resistance to one or more US soil-borne pathogens, but not to significant reniform resistance. In LONREN lines, replacement of the endogenous *G. hirsutum* allele by *Ren^{lon}* compromises resistance to the corresponding US soil-borne pathogen(s), and leads to stunting in fields that harbor both the pathogen(s) and nematode.
2. Linked resistance gene loss: Replacement of a *G. hirsutum* c11 segment by a homeologous *G. longicalyx* segment entails loss of one or more nearby (clustered or linked) endogenous genes that contribute significantly to resistance to soil-borne pathogens. Linked gene(s) could also impart greater sensitivity to herbicides.
3. Gain of the linked gene(s) that exacerbate sensitivity to one or more soil pathogens: Replacement of a *G. hirsutum* c11 segment by a homeologous *G. longicalyx* segment entails gain of one or more nearby genes that exacerbate sensitivity to one or more soil pathogens.
4. Pleiotropic effect of *Ren^{lon}*: The *G. longicalyx* allele, *Ren^{lon}* has dual effects, one being higher resistance to reniform nematodes and another being reduced resistance to certain other soil-borne pathogens.
5. Secondary effect of resistance: The host plant resistance to reniform nematode leads to extensive tissue damage, “hyper-sensitivity”, which compromises root vigor and viability. Exaggerated loss of key tissues in LONREN lines at nematode feeding sites, such as epidermis, could increase susceptibility to certain soil-borne pathogens as a secondary effect.

6. Combined effects: Root damage from hypersensitivity to reniform nematodes and reduced genetic resilience to soil-borne pathogens (174).

Recent evidence indicates that LONREN germplasm is differentially sensitive to high-nematode populations, and suffers considerable root damage, which contributes to stunting (136). Controlled experiments indicate genotypes with the *Ren^{lon}*-bearing segment are also differentially susceptible and/or sensitive to specific soil-borne fungi, including *Thielaviopsis basicola* (12). The increased susceptibility could be due to overly hypersensitive reaction to reniform nematodes and (or) nearby alien genes co-introduced by “linkage drag” that modify resistance to one or more soil fungi.

Recombining *Ren^{lon}* into c21 rather than c11 did not avoid stunting, and indicated that loss of native c11 resistance genes does not account for the stunting. Newly created hybrids between BARBREN and LONREN show LONREN-like stunting levels, and indicate the LONREN factor(s) that lead to stunting are dominant and (or) epistatic to the non-stunting nature of BARBREN713 resistance.

It would seem improbable, but possible that the gene for nematode resistance directly affects multiple traits, so indirect effects seem much more likely, e.g., due to tissue damage from hypersensitive cell death, or accentuation of the downstream pathogen response system(s). For example, allelic choice at the methylation of the benzoxazinoids locus can favor resistance to aphids or caterpillars (102). Recent literature has documented physiological connections between resistance to nematode and other

pathogens, ramifications of which can be pathogen-specific (57, 107). Also, closely linked genes would be expected to affect multiple traits, including pathogen resistance and agronomic performance and quality. Such effects could be due to direct effects of specific genes on specific pathways and traits, while others might be quite indirect. Of special interest may be other genes that affect resistances to other pathogens, because many genes important to resistances are known to be clustered and so it would be quite likely that the gene for reniform resistance is flanked by other *G. longicalyx* genes that alter the profile of resistances to multiple pathogens. Bell et al. noted that FOV and *Thielaviopsis* were synergistically detrimental in causing severe stunting in concert with the reniform nematode (12). Alterations in pathogen resistance could indirectly affect numerous other agronomic traits, including herbicide sensitivity. In any case, high-resolution recombination offers the simplest approach to selecting reniform-resistant cottons that are free of unwanted genetic effects of linked alien genes. Products from recombination would not be GMO, and would be readily bred and deployed in US grower fields. The main objectives of my work have been: [1] to develop *Ren^{lon}*-linked markers and map the *Ren^{lon}* region at high resolution, [2] to recombine *Ren^{lon}* and nearby loci and determine if “stunting” is due to linkage drag, [3] to create genomic and germplasm resources for genetic manipulation of chromosome-11, especially *Ren^{lon}*, and [4] to determine if "stunting" is due solely to plant-nematode interactions or involves additional pathogens.

CHAPTER II

DEVELOPMENT OF SNP MARKERS BETWEEN *G. longicalyx* AND *G. hirsutum*

BY RNA-SEQ

Introduction

Next-generation sequencing (NGS) technologies are being extensively used for genome-wide genetic marker development through RNA-seq, reduced-representation sequencing, restriction-site-associated DNA sequencing (RAD-seq) and low-coverage genotyping (43). The availability of abundant markers will facilitate association mapping, fine-mapping of regions of interest, marker assisted selection (MAS) to circumvent the problem of linkage drag during introgressions or map-based cloning. The most common application of NGS is SNP discovery.

In recent years, SNP markers have gained much interest in the scientific and breeding community (116). They occur in large numbers as differences of individual nucleotides between individuals and every single-copy SNP is a potentially useful DNA marker. Recent emergence of NGS technologies such as 454 Life Sciences (Roche Applied Science, Indianapolis, IN), HiSeq (Illumina, San Diego, CA), SOLiD and Ion Torrent (Life Technologies Corporation, Carlsbad, CA) have eliminated the problems associated with low-throughput and high-cost SNP discovery (96). RNA-seq can provide the most informative SNPs for gene synteny-based comparative genomics and association

mapping (64, 105, 162, 167). A number of software programs have been developed for SNP identification from the NGS data (89, 147).

Transcriptome sequencing using NGS technologies allows rapid and inexpensive SNP discovery within genes and avoids highly repetitive regions of a genome. While SNP discovery in crops with simple genomes with a high quality reference genome sequence such as rice and arabidopsis (106, 169) is a relatively straightforward process, complex genomes pose serious obstacles for the researchers interested in developing SNPs. One of the major problems is the highly repetitive nature of larger complex plant genomes (103). Effective SNP discovery in complex genomes requires additional analysis to consider duplicate loci and to identify and eliminate pseudo-SNPs produced by mis-assembly of paralogous and homoelogenous sequences inherent to polyploidy genomes. Prior to the emergence of NGS technologies, researchers relied on different experimental strategies to avoid repetitive portions of the genome. These include discovery of SNPs experimentally by resequencing of unigene-derived amplicons using Sanger's method (166) and *in silico* SNP discovery through the mining of SNPs within EST databases followed by PCR-based validation (8).

The cotton genus (*Gossypium* L.) consists of five allotetraploid ($2n = 4x = 52$) and 45 diploid ($2n = 2x = 26$) species (56). There are four domesticated species, including two New World allotetraploids, *G. hirsutum* and *G. barbadense*, and two Old World diploids *G. arboreum* and *G. herbaceum*. Within these 4 cultivated species, Upland Cotton, *G.*

hirsutum L. (AD1) is the most important fiber crop in the world. The diploid species fall into eight cytological groups, or ‘genomes’, designated A–G and K based on the chromosome-pairing relationships (51). *Gossypium longicalyx* (F1) is a wild species, originally from Africa. It has reniform nematode resistance (170) and potential fiber quality traits (46, 160). The first genome sequenced from the *Gossypium* genus was *G. raimondii* (2n=26, D5 genome), a Peruvian tree. This genome was sequenced with a combination of Sanger, Roche 454 pyrosequencing and Illumina paired-end reads (111).

In present study RNA-seq was performed using *G. longicalyx* and five different cultivars of *Gossypium hirsutum*, ‘Acala’, ‘FM832’, ‘Sealand’, ‘PD-1’, and ‘TM-1’ using the Illumina NGS platform to identify SNPs between *G. hirsutum* and *G. longicalyx* (*Gh_Gl* SNPs). It is well known that cotton improvement efforts are seriously constrained by the limited amount of genetic diversity available to breeders and the *Gh_Gl* SNPs produced from this project will be useful for future introgressions from *G. longicalyx*, genome-wide mapping, and fine-mapping in specific regions of the genome.

Here, I am reporting the following SNP development information as conducted in conjunction with this whole LONREN project. Two methods were used for developing *Gh_Gl* SNPs by collaborators from the University of California-Davis. Method I: Use assembly from RNA-seq of five *G. hirsutum* cultivars was used as the reference to detect *Gh_Gl* SNPs. Method II: A reference assembly built from RNA-seq of TM-1 (based on Roche 454 sequencing reads) and public ESTs was used as a reference to develop *Gh_Gl*

SNPs. A total of 68,578 *Gh_Gl* SNPs were developed by Method I and 38,217 *Gh_Gl* SNPs were developed by Method II. A subset of 86 randomly selected *Gh_Gl* SNPs by Method I was validated by KASPar assays (<http://www.lgcgenomics.com/>).

Approximately 90% of the SNPs were successfully developed to KASPar assays. 78% of the total *Gh_Gl* SNPs were mapped to *G. raimondii* D5 genome.

Materials and Methods

Sequencing and informatic analyses leading to SNP development was done in collaboration with several researchers in the Stelly laboratory at Texas A&M University and the Allen Van Deynze laboratory at UC Davis. Dr. Hamid Ashrafi and Dr. Allen Van Deynze of UC Davis completed the assemblies, wrote necessary program scripts, and made most of the comparisons used for discovery of putative SNPs. Illumina sequencing was performed at the UC Davis Genome Center.

Plant material, RNA extraction and library preparation

Five *G. hirsutum* cultivars – ‘Acala Maxxa’ (Acala), ‘FiberMax 832’ (FM832), ‘Sealand 542’ (Sealand), ‘PD-1’, ‘TM-1’ and *G. longicalyx* (plant number 200908137.04 from the Beasley Lab collection planted at Texas A&M University) were used for transcriptome analysis. Young leaf tissues were sampled from each plant and used to isolate total RNA using the Qiagen RNeasy Mini Kit per manufacturer instructions. RNA isolates were quantified using NanoDrop spectrophotometry and checked for quality by gel electrophoresis. Illumina TruSeq[®] RNA libraries were prepared using the manufacturer

protocol. Libraries were normalized (98) by denaturation and rehybridization in NaCl and TMAC (tetramethyl ammonium chloride) buffers and were then treated with Duplex Specific Nuclease to digest any remaining DNA contamination (175). The treated library was re-amplified using the Illumina library primers via PCR per manufacturer's instructions. The libraries were sequenced using the Illumina Genome Analyzer II for 85 cycles to get paired-end reads.

De novo assembly of five G. hirsutum cultivars

For each *G. hirsutum* cultivar, a non-redundant set of sequences was obtained using the V-match program in the Velvet assembler (**Fig. 2.1**). One iteration of assembly for each cultivar was also created using the CLC Genomics workbench (V. 4.0). Multiple iterations (from 21 K-mers to 51 K-mers) of assembly were created using Velvet assembler for each cultivar. And a non-redundant set of sequences for each cultivar were obtained by V-match program (V. 2.0) (<http://www.vmatch.de/>). The assemblies obtained by the two independent programs were combined to create a Velvet-CLC assembly from each cultivar. A final *G. hirsutum* assembly was obtained by combining each of the five Velvet-CLC assemblies in the program CAP3 (**Fig. 2.1**).

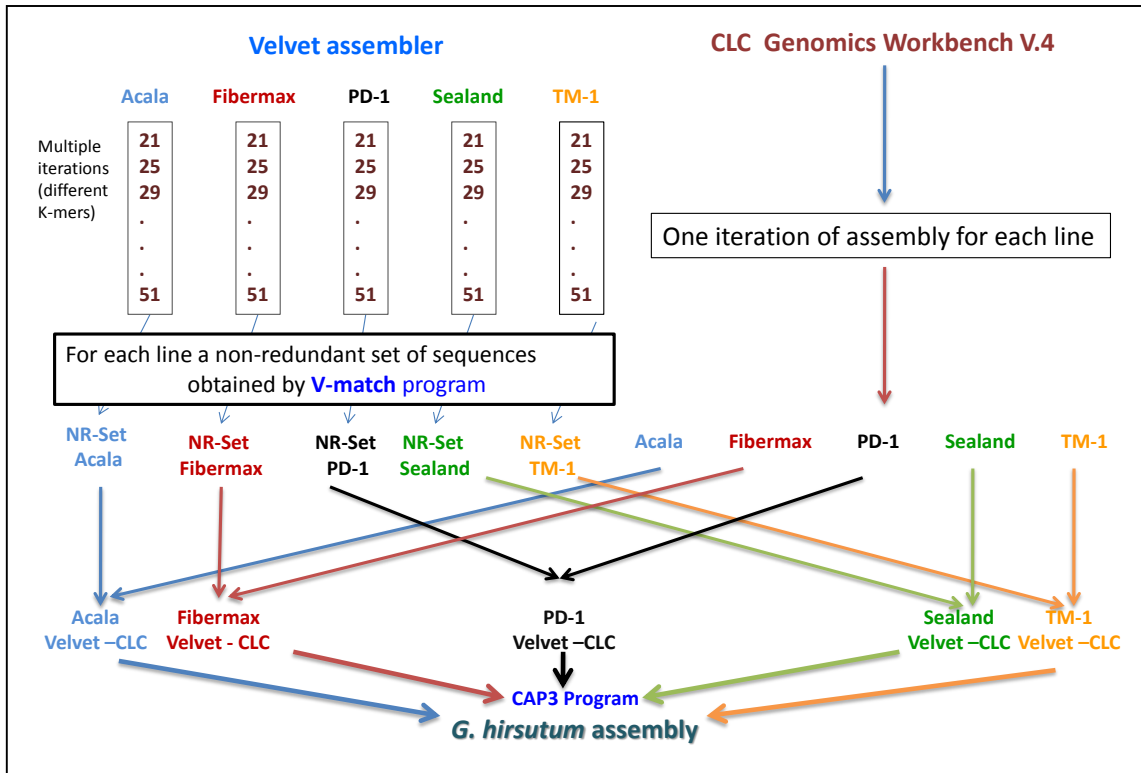


Figure 2.1. Assembly strategy for *G. hirsutum* transcriptome reference assembly.

Identification of SNPs

Two methods were used separately to identify the single nucleotide variations between *G. longicalyx* and *G. hirsutum* cultivars.

Method I: The *G. hirsutum* assembly derived from the five cultivars (**Fig. 2.1**) was used as a reference and the RNA-seq reads of *G. longicalyx* were mapped to this assembly using the Burrows-Wheeler Alignment tool (BWA) for the detection of SNPs and INDELS. To map all the reads of each *G. hirsutum* cultivar separately to the *G. hirsutum* assembly, the SAMtools program was used with the merge option set to merge all alignment files and generate a SAM pileup file for all cultivars together as well as

generate a pileup file for each individual cultivar. Galaxy and custom Perl scripts were used to parse the SAM pileup files and to generate a so called 'genotype' table. From the genotype table the SNPs between *G. hirsutum* and *G. longicalyx* (*Gh_Gl* SNPs) were identified by custom Perl algorithm(s). For each contig in the SNP file, the full length sequence of the contig was extracted from the assembly file and SGN (<http://solgenomics.net/>) was used to find the putative intron position(s). From the list of SNPs, 50 bases from both sides of the SNP site were extracted from the reference sequence. The list of SNPs was reduced to SNPs not in the vicinity of 100 bases of an intron.

Method II: Reads from *G. longicalyx* were trimmed for quality and then aligned to the *Gossypium hirsutum* assembly created from inbred TM-1 (Ashrafi et al. unpublished), using the CLC Genomics Workbench (V. 5.0). The mapping files were exported as *BAM* files to a Linux server and SAMtools was used to call variants. The resulting pileup files were filtered using the filter pileup perl script in Galaxy (<https://main.g2.bx.psu.edu/>) to remove INDELS and positions with coverage less than three. The resulting file was then further filtered using an in-house perl script which required the two genotypes to be homozygous for different bases with minimum coverage of 10. Putative SNPs were then removed from the list if they were located within 50 bases of a predicted intron-exon boundary on the TM-1 assembly using SGN (<http://solgenomics.net/>). The list of SNPs was reduced to SNPs not in the vicinity of 100 bases of an intron.

From the last SNP list (step above) and list of GSPs (Genome Specific Polymorphisms) or INDEL positions, we defined three classes of SNPs:

- Class I contained SNPs in contigs with no GSPs or INDELS;
- Class II contained SNPs with no GSPs or INDELS in the vicinity of 50 bases of the SNPs, but beyond this 50bp range, GSPs or INDELS were allowed;
- Class III contained SNPs with one GSP or INDEL within 50 bases of the SNP.

SNP validation

Prior to SNP applications, the SNPs were validated to identify true SNPs from random sequence errors. To evaluate the sets of putative SNPs identified by a given bioinformatic "pipeline", a subset of the overall set was analyzed experimentally in a wet lab to estimate the rate at which the set of putative SNPs would convert to functional assays. LGC Genomics/ Kbioscience KASPar assays (<http://www.lgcgenomics.com/>) were used. Allele-specific and allele-flanking primers were developed using BatchPrimer3 using an optimal primer T_m of 57°C (minimum 55°C, maximum 60°C, maximum difference between primers 5°C), optimal product size of 50 base pairs (minimum 50 base pairs, maximum 100 base pairs) and the default settings were used for the remaining parameters. KASPar assay primers were mixed at the dilutions specified by LGC Genomics then assessed using a small cotton screening panel.

A subset of 86 *Gh_Gl* SNPs was randomly selected from SNPs identified by Method I. The DNA samples (2 μ L at 10ng/ μ L) were formatted in 96-well plates. They were dried at 60-65°C for an hour. The screening panel included Acala, FM832, Sealand, PD-1, TM-1 and *G. longicalyx*. Each genotype was replicated three times. Reagents included sterile deionized water 3.826 μ l, 2x Reaction mix 4 μ l, Primer mix 0.11 μ l, 50mM MgCl₂ 0.064 μ l. A total of 8 μ l of reagents was added to each well of the plate that contained the dried DNA samples and then sealed by Flexiseal with sealing tape at 175°C. The PCR was conducted by the KASPar assay (KBioscience Ltd., Hoddesdon, UK), following an acclimation step of 94°C for 15 minutes, the first 10 cycles consisted of: denaturation at 94°C for 20 seconds, followed by annealing starting at 65°C for 1 minute, decreasing 0.8°C per cycle to an annealing temperature of 57°C for the final cycle. This was followed by 28 cycles of denaturation at 94°C for 20 seconds, and annealing at 57°C for 1 minute. Plates were then briefly centrifuged, then read on the Pherastar plate reader. The Pherastar files were imported into KlusterCaller software for genotyping. In the event that greater amplification was required, plates were returned to the hydrocycler for iterations of 3 additional PCR cycles, with the denaturation steps at 94°C for 20 seconds, and annealing at 57°C for 1 minute.

Alignment of SNPs to the D5-reference genome

The SNP and flanking sequences were aligned to the *Gossypium raimondii* (D5) reference genome sequence (111) using BWA in Galaxy using default settings. SNPs were separated into files based on D5-genome scaffold alignment. Scaffold files were

sorted by genome position. Density of markers along the D5 genome scaffold were plotted in the R program using a sliding window over genome position.

Results

Plant material and sequencing

Five *G. hirsutum* cultivars ‘Acala’, ‘FM832’, ‘Sealand’, ‘PD-1’, ‘TM-1’ as well as *G. longicalyx* were used for RNA sequencing. A few small leaves were used for each RNA extraction. cDNA libraries to be used for sequencing were prepared from the poly-A containing RNA and sequenced on the Illumina Genome Analyzer using a 101 bp paired-end strategy.

RNA-seq libraries of the five *G. hirsutum* cultivars were run in 31 lanes separately of the Illumina flow cell whereas the *G. longicalyx* sample was run in 6 lanes. Paired-end sequencing of each transcriptome generated 53,468,192, 52,257,485, 45,278,905, 50,458,314 and 60,237,336 sequence reads for Acala, FM832, Sealand, PD-1, TM-1, respectively, whereas 52,050,305 sequence reads were generated for *G. longicalyx* (**Table 2.1**).

Table 2.1. Sequencing statistics of five *G. hirsutum* cultivars and *G. longicalyx*.

Species	Cultivar(s)	Genome	No. of Illumina Lanes for each cultivar	No. Total Reads	Percent Reads Used*	Percent Reads Used
					Velvet Assemb.	CLC
<i>G. hirsutum</i>	Acala Maxxa	(AD) ₁	31	53,468,192	4%-40%	58.8%
	FiberMax 832	(AD) ₁	31	52,257,485	4%-38%	58.3%
		(AD) ₁	31	45,278,905	5%-38%	57.3%
	PD-1	(AD) ₁	31	50,458,314	6%-39%	58.7%
	Sealand 542	(AD) ₁	31	60,237,336	1%-43%	41.1%
	TM1			TOTAL =>	261,700,232	2%-29%
<i>G. longicalyx</i>	-	(F ₁)	6	52,050,305	0.7%-57%	43.8%

* Percent reads used in the assembly by Velvet when using different K-mers from 21-51.

De-novo assembly of the transcriptome

Cleaned reads were assembled using the Velvet *de novo* assembly program and the CLC Genomics Workbench. Reads were assembled for different K-mer values (K21 to K51) in Velvet and one iteration of assembly for each cultivar in the CLC Workbench. The assembled data were analyzed for the total number of contigs produced by each program, N50 values, and number of contigs greater than 300 nt (**Table 2.2**).

Table 2.2. Assembly statistics of five *G. hirsutum* cultivars and *G. longicalyx*.

Species,	Cultivar(s)	No. of Velvet Contigs > 300 nt	Total No. Nuc. Velvet	N50	No. of CLC Contigs > 300 nt	Total No. Nuc.	N50	No Contigs	Total No. Nuc.	N50
<i>G. hirsutum</i>	Acala Maxxa	86,127	49,466,603	597	35,558	21,714,353	661	-	-	-
	FiberMax 832	84,773	48,471,346	596	34,606	21,079,331	656			
	PD-1	77,647	43,570,663	579	32,549	19,387,564	636			
	Sealand 542	83,896	47,832,219	593	34,393	20,906,291	653			
	TM1	104,986	55,970,600	545	36,099	17,363,787	479			
Mega Assembly (Velvet + CLC)	-	-	-	-	-	-	-	71,916	61,283,682	1145
<i>G. hirsutum</i>										

Identification of SNPs

The BWA program was used for identifying SNPs between *G. longicalyx* and *G. hirsutum*. Two categories of *Gh_Gl* SNPs were generated by Method I (*G. hirsutum* assembly served as the reference): Non-specific *Gh_Gl* SNPs, which are the SNPs between *G. longicalyx* and one of the five *G. hirsutum* cultivars; Specific *Gh_Gl* SNPs, which are the SNPs between *G. longicalyx* and all five *G. hirsutum* cultivars. Fifty bases on each side of the SNP site were extracted from the reference sequence. Overall, 68,578 SNPs were identified between *G. longicalyx* and *G. hirsutum* using Method I which were classified as either "Nonspecific" or "Specific", and then further classified as belonging to Class I, II, or III as described above (**Additional file 1**).

Gh_Gl SNPs between *G. longicalyx* and TM-1 were generated by Method II (TM-1 assembly and public ESTs were used as reference). Using this method, 38,217 SNPs were identified between *G. longicalyx* and TM-1 which were classified as Class I, II, or III (**Additional file 1**). **Table 2.3** shows the distribution of SNPs identified using both methods as well as their class type.

Table 2.3. Summary of *Gh_Gl* SNPs identified by the two methods.

Method	Specificity	Class type*	#SNPs
I	Non-specific	I	12,119
		II	7,364
		III	1,709
	Specific	I	28,176
		II	15,666
		III	3,544
II	<i>G. longicalyx</i> vs. TM-1	I	14,546
		II	18,960
		III	47,11
* Class I (Clean SNPs) – No intron, no INDEL and no GSP in the SNP-containing contig. Class II – No intron, no INDEL and no GSP within 50 bases of the SNP. Class III – No intron within 100 bases of SNP, but one GSP/INDEL within 50 bases of the SNP.			

SNP validation

Randomly selected SNPs from non-specific and specific types, including all three classes, were tested by KASPar assay. Overall, 86 SNP-based markers were tested. Approximately 90% of the *Gh_Gl* SNPs tested were convertible to KASPar assay (**Table 2.4**).

Table 2.4. *Gh_Gl* SNP validation summary.

Specificity	Class type*	Number of SNPs tested	Successful SNPs	Rate of success
Non-specific	I	14	12	
	II	14	13	
	III	15	14	
Specific	I	14	14	
	II	14	12	
	III	15	13	
Total	I, II and III	86	78	90.70%

* Class I (Clean SNPs) – No intron, no INDEL and no GSP in the SNP-containing contig.
Class II – No intron, no INDEL and no GSP within 50 bases of the SNP.
Class III – No intron within 100 bases of SNP, but one GSP/INDEL within 50 bases of the SNP.

Alignment of SNPs to the D5 cotton genome

The overall D5 genome assembly (V2.1) includes 748.1 Mb, with 1,033 scaffolds and 19,735 contigs. The scaffold N50 = 6 (62.2 Mb), contig N50 = 1,596 (135.6kb), and 41 scaffolds are > 50kb in size. Overall the assembly putatively represents approximately 99.0% of the genome (<http://www.phytozome.net/cotton.php>).

In total 106,795 *Gh_Gl* SNPs (78%), including 68,578 *Gh_Gl* SNPs developed by Method I and 38,217 by Method II, were aligned to the D5 genome assembly using the BWA program with default settings (**Table 2.5**).

Table 2.5. Numbers and classifications of the *Gh_Gl* SNPs mapped to the D5 genome.

Specificity	Class type*	Unmapped	Mapped	Total
Non-specific	I	1943	10176	12119
	II	1103	6261	7364
	III	347	1362	1709
Specific	I	6033	22143	28176
	II	3153	12513	15666
	III	1067	2477	3544
G. longicalyx vs. TM-1	I	3216	11330	14546
	II	4130	14830	18960
	III	1614	3097	4711
	Total	22606	84189	106795
	Percent	21.17	78.83	
* Class I (Clean SNPs) – No intron, no INDEL and no GSP in the SNP-containing contig. Class II – No intron, no INDEL and no GSP within 50 bases of the SNP. Class III – No intron within 100 bases of SNP, but one GSP/INDEL within 50 bases of the SNP.				

Of the markers aligned, 99.75% were aligned to 1 of the 13 pseudo-chromosome scaffolds. Only 267 markers were aligned to unplaced scaffolds (**Table 2.6**). All the *Gh_Gl* SNPs with the map position on D5 genome were shown in the **additional file 1**.

Density of markers along the D5 genome 13 major scaffolds were plotted in the R program using a sliding window over genome position.

Table 2.6. Distribution of mapped *Gh_Gl* SNPs.

Scaffold Name	Number of Hits	Percent of Total Hits
Unmapped	22606	21.17
Chr01	6445	6.03
Chr02	5734	5.37
Chr03	4309	4.03
Chr04	6607	6.19
Chr05	6168	5.78
Chr06	6422	6.01
Chr07	8616	8.07
Chr08	7349	6.88
Chr09	10643	9.97
Chr10	5607	5.25
Chr11	5776	5.41
Chr12	4236	3.97
Chr13	6010	5.63
other scaffolds	267	0.25

The density of markers along the 13 major scaffolds of the D5 genome were plotted in R using a sliding window over genome position in collaboration with K Hoegenauer (**Fig.**

2.2). The mapped SNPs were not uniformly distributed across each chromosome. More SNPs were clustered in gene-rich, high-recombination euchromatic regions than in repeat-rich, low-recombination heterochromatic regions.

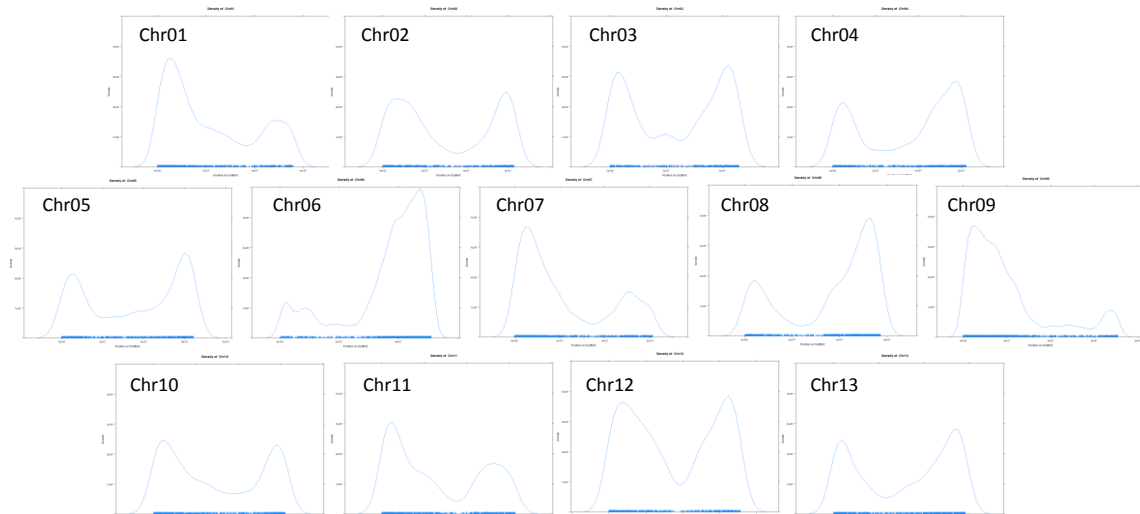


Figure 2.2. Distribution of SNPs relative to *Gossypium raimondii* (D5) draft genome version 2.1.

Discussion

Apart from the initial bottleneck encountered during the domestication process, cotton breeding has frequently involved crossing and re-selection within small sets of breeding materials which has led to the loss in genetic diversity (25, 28, 100, 163). The narrow genetic base of Upland cotton has become a serious concern since limited genetic diversity translates to limited allelic availability for continued genetic gain (27). With a heightened risk of genetic vulnerability to disease epidemics and climate change, elite

breeding programs could benefit from the unexploited standing genetic variation of obsolete cultivars without the yield drag typically associated with wild accessions. Interspecific gene transfer through sexual hybridization and introgression continues to be the predominant method for introducing new alleles into many domesticated plant species. The genes other than the targeted gene on the alien chromatin usually cause linkage drag, a deleterious effect on yield and quality. Reduction of linkage drag is the most difficult goal to achieve because of the selection for the target. Hence, this is where use of marker-assisted selection (MAS) is most rewarding (69, 171). Combined with traditional selection techniques, MAS has become a valuable tool in selecting organisms for traits of interest, such as color, meat quality, or disease resistance.

Such is the case with LONREN which was created to confirm reniform resistance found in *G. longicalyx* (127). Combining *G. longicalyx* with *G. hirsutum* offers superior fiber trait characteristics as well (160). The large number of *G. longicalyx* markers developed here will provide tags for a large number of genes in which differences exist between *G. hirsutum* and *G. longicalyx*. All of these *Gh_Gl* SNPs will be available for genome-wide mapping, fine-mapping in specific regions of the genome and future introgressions from *G. longicalyx*. In addition, 86 markers across the genome were randomly selected and ~90% were validated by KASPar assay.

Of the total 106,795 *Gh_Gl* SNPs, only 22,606 of them did not map to D5 genome, whereas over 78% of them did map, even though the F1 and D5 genomes are not

evolutionarily close. The A-genome diploids native to Africa and the Mexican D-genome diploids diverged ~5-10 MYA (135), and several lines of evidence indicate that *G. longicalyx* from Africa with its F-subgenome is more closely related to the A-subgenome than the D-subgenome (112).

The *Gh_Gl* SNPs were aligned to the D5 genome to obtain some indication of the relative position of the SNPs in the genome. As expected due to the nature of heterochromatic regions near centromeres and telomeres, the SNPs were distributed unevenly across the 13 chromosomes. A bimodal pattern was evident, where large numbers of SNPs mapped near the sub-telomeric regions of chromosome arms while small numbers of SNPs mapped to what are inferred to be centromeric and telomeric regions, i.e., approximately the middle and ends of the reference scaffolds, since most cotton chromosomes are metacentric or submetacentric (93). However, based on a sliding-window approach, the diminishing SNP densities in telomeric regions are difficult to interpret, because they inferably include the combined effects of a statistical artifact, and the reduction and eventual absence of genes in the sub-telomeric and telomeric regions. The statistical artifact would arise as the “window” progressively includes the end of a chromosome.

CHAPTER III
NON-DESTRUCTIVE HIGH-THROUGHPUT DNA EXTRACTION AND
GENOTYPING METHODS FOR COTTON SEEDS AND SEEDLINGS

Introduction

Molecular plant breeding requires the genotyping of a large number of individuals for diversity analysis, marker-assisted selection, mapping and "fingerprinting". For high-throughput genotyping programs, the most limiting factor for the use of PCR amplification in analyzing large plant populations is the time and expense of extracting DNA.

There are several reports on rapid extraction of DNA from plant tissues, but almost all of these involve tissue from whole plants (17, 50, 60, 86). In addition, several in-house DNA extraction protocols have been described for temperate crops, based on the use of 96-well Microliter microtiter plates (49, 109).

DNA isolation from cotton (*Gossypium sp.*) is complicated by the presence of phenolic compounds (110). During tissue homogenization of certain plants, phenolics become oxidized and irreversibly bind with proteins and nucleic acids (91). This irreversible binding produces a gelatinous material, which is hard to separate from organelles, and which renders DNA unsuitable for PCR or restriction enzyme digestion (114).

Previously developed protocols for extraction of cotton DNA suitable for PCR are based

on leaf tissue, and involve the use of many chemicals (34, 88, 110). A 96-well plate based high-throughput DNA extraction method was reported by Xin et al. (168). It is applicable to many species, including cotton, and avoids grinding and/or the use of organic solvents, but it relies on plant leaf tissue.

DNA extraction performed on seed or cotyledon instead of leaf tissue would allow genotyping to be carried out independently of the growth season, or very early in the growth season. This would enable seed to be selected prior to planting or cotyledons prior transplanting. In addition, it would be useful for identifying rare recombinants or gene combinations from among thousands of individual seed or seedlings. DNA extraction performed on seed has been described previously using single dry seed of wheat, rice, barley and several other species (3, 35, 75), including patented robotics-enabled methods (47, 70).

In this chapter, I report on the development of a high-throughput method for non-automated but rapid extraction of DNA from ungerminated seeds or cotyledons suitable for PCR analysis. This method was derived in part from the methods described by Xin et al. (2003) and von Post et al. (2003) (152, 168). Relative to commercial kits readily applicable to cotton seeds and cotyledons, this method is a magnitude lower in cost and more time saving. Being applicable to seeds and cotyledons, it enables genotyping before planting or during initial seedling stages, both of which offer numerous advantages. This method has already proven itself useful in multiple ongoing research

projects that require low-cost DNA preparations for PCR-based applications.

Preliminary tests indicate that with minor adjustments, it is applicable to seeds of other plants with moderate or large seed size.

Materials and Methods

Solutions and buffers

Buffer A (100 mM NaOH, 2% Tween[®] 20) must be made fresh from 10 M NaOH and 20% Tween 20 stock solutions just before use. For buffer B [100 mM Tris-HCl (Sigma), 2 mM EDTA], the pH is ~2.0. Buffer B does not have to be made fresh for each extraction and can be stored at room temperature (152, 168).

Plant materials

Mature undelinted and delinted seed were used to assess applicability to seed of cotton (*G. hirsutum*). Cotyledon tissues of cotton were grown under greenhouse conditions (Day Temperature: 29.4°C, Night Temperature: 23.9°C, Humidity: 70%, Light: Natural light) in a commercial growing medium (Metro-Mix 700).

Cotton seed and seedling tissue sampling procedures

Delinted or undelinted seed were sanded opposite the hypocotyl using the Sanding Drum (Dremel #407) with 60-grit Sanding Bands (Dremel #408), just far enough to expose cotyledonary tissue (**Fig. 3.1a**). Sanded seeds were inserted one seed per well into modified PCR plates (Phenix #MPS-499) labeled with a unique identifying code (**Fig.**

3.1c), from which the end of each well had been previously removed (e.g., cut away with a box-cutter or similar tool) (**Fig. 3.1b**), then covered with adhesive tape (e.g., packing tape) to prevent the seed from falling out of the wells. When seed are variable in size, additional manual pressure may be required to force seed into the well, or a water-soluble glue may be applied to the plate to more firmly secure the smaller seed. The PCR plate was then inverted, and an Engraving Cutter (Dremel #107) was rotated at a slow speed while inserting it to the depth of the cutting head (approximately 2.4 mm) (**Fig. 3.1d**), such that the abrasion created fine tissue particles in each well. After each use, the cutting head was cleaned by drilling a hole into a conventional pencil eraser (**Fig. 3.1e**). About 3.5 mg of particulate cotyledonary tissue was obtained from each sample. For applications involving smaller seeds, or those requiring smaller amounts of DNA, a 1.6 mm Engraving Cutter (Dremel #106) was used, which yielded around 1.5 mg of tissue. Once drilling was completed, a regular PCR plate (Phenix #MPS-499) labeled with a matching unique identifying code was physically placed male-to-female with the modified collection/storage plate (**Fig. 3.1f**), and then the pair of plates was inverted and slapped to dislodge and transfer the drillings into the matching wells of the regular PCR plate. The modified plates of sampled seeds were stored sequentially according to labels. After genotyping, seed were selected based on genotype, then extracted from the plate and germinated in "rag dolls" of germination paper or in Jiffy peat pellets.

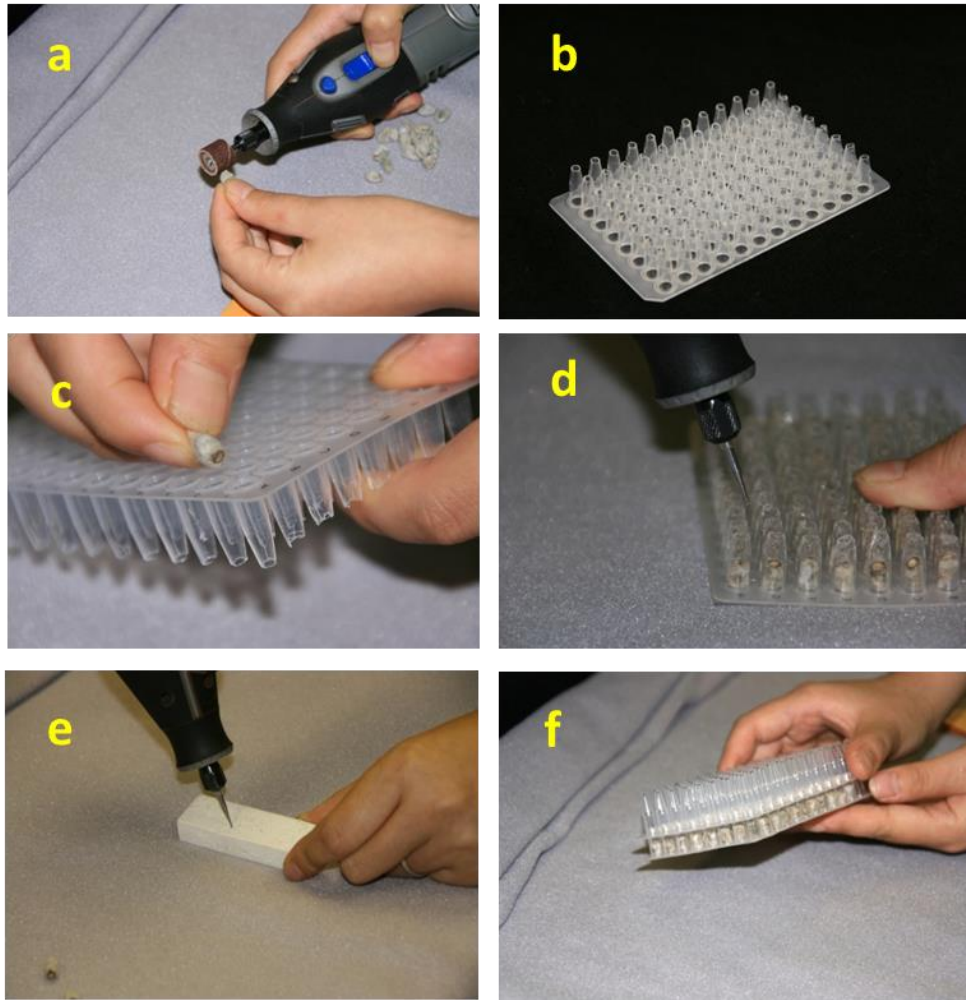


Figure 3.1. Cotton seed tissue sampling process. (a) Sand undelinted cotton seed using a Sanding Drum. (b) Modify a PCR plate by cutting tips of wells. (c) Place sanded seed into modified PCR plate. (d) Drill sanded seeds using an Engraving Cutter. (e) Clean Engraving Cutter by drilling into an eraser. (f) Match wells of modified (seed) and unmodified (sample) plates, then invert and hand-slap upper plate to dislodge and transfer tissue from modified to unmodified plate.

Seedling tissue samples were obtained from 3-day post-emergence cotyledons (**Fig. 3.2a**) using a standard (6 mm diameter) single-hole punch (**Fig. 3.2b**), which was wiped clean between samples. For applications requiring smaller amounts of DNA, a commercially available 3 mm punch, or alternatively, a 200 μ L pipette tip can be cut to

the appropriate size, and used to create 3 mm diameter leaf disks. Each tissue disk was transferred into a well of a 96-well plate (**Fig. 3.2c**) or a flat-bottomed microfuge tube, depending on the number sampled overall. Samples were dried by one of two methods before extraction. Either tissue samples were heated in a dry incubator for 1 hour at 60°C, or desiccant beads were added to the tubes/wells, closed, and allowed to dry for 24 hours. Once dried, tissues were crushed individually to increase contact surface; a disposable or clean-able implement was used to avoid cross-contamination.

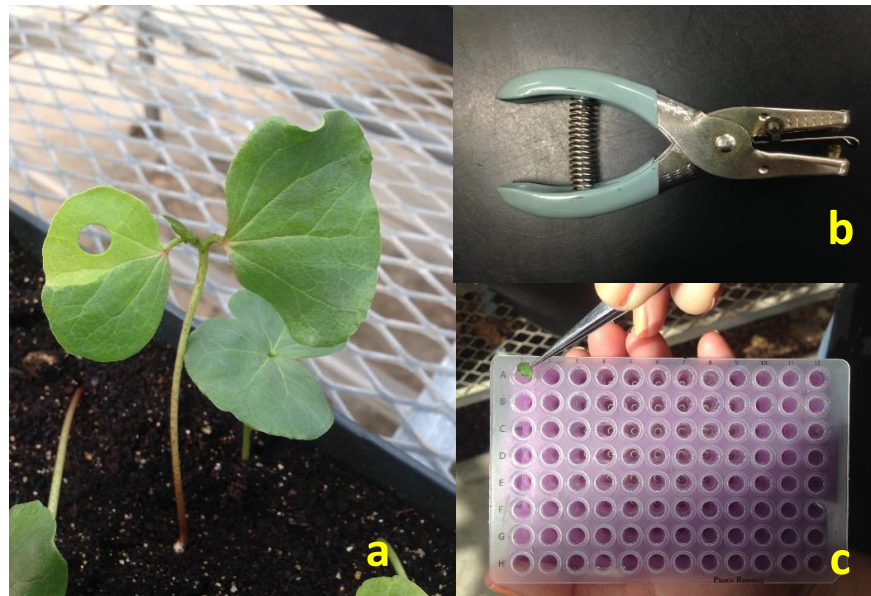


Figure 3.2. Cotyledon tissue sampling process. (a) Obtain seedling tissue samples from 3-day post-emergence cotyledons. (b) A standard (6mm diameter) single-hole punch. (c) Transfer cotyledon tissue disk into a 96-well PCR plate.

DNA extraction

50 μL of Buffer A was added to each sample and plates were optionally mixed or vortexed and briefly centrifuged to ensure reagent contact with the seed tissue or cotyledon disk. Plates can be processed simultaneously either by placing in 1200W microwave for 1 minute at 30% power (approximately 360W) or by using a bench-top oven, water bath or thermocycler set at 95°C for 10 minutes. Plates were removed and 50 μL of Buffer B was added to each sample. Then plates were diluted 2x with de-ionized H₂O, mixed or vortexed and briefly centrifuged to consolidate extract. Ten μL of supernatant was transferred to another plate, and the new plate was then diluted another 10x with de-ionized H₂O for seed DNA extraction. For cotyledons, after Buffer addition and heating steps, 10 μL was transferred into a new plate, then the new plate was diluted 10x directly. For PCR-based analysis, approximately 2 μL of the diluted extract was transferred from a DNA plate to a PCR plate using either a conventional multi-channel pipette or i-Pipette (Apricot Designs). The whole procedure is shown in **Table 3.1**.

Table 3.1. Procedure of high-throughput DNA extraction from cotton seeds or seedlings.

1. Add 50 μ L Buffer A to the samples, then seal the plate.
2. Mix to immerse tissue, then centrifuge briefly to consolidate tissue.
3. Heat, using one of several options: (1). Microwave for 1 minute at 30% power (approximately 400 Watts); (2). Heat for 10 minutes at 95°C in an oven, a thermocycler or a waterbath.
4. Add 50 μ L Buffer B to the plate.
5. Mix or vortex, then centrifuge briefly to consolidate extract and cellular debris.
6. For cotton seed extract: dilute 2x, transfer 10 μ L to a new plate, and dilute new plate 5x or 10x. For seedling extract: transfer 10 μ L to a new plate, then dilute DNA samples in the new plate 10x.
7. Transfer 2 μ L of the diluted extract to a new PCR plate. Optionally dry the samples at 65°C.
8. Conduct PCR.

Primers

Two cotton SNPs were used in this study, one for testing seed-based DNA extraction, and the other for seedling-based extraction. The SNPs were chosen from subsets of large numbers of interspecific SNPs identified by comparative RNA-seq analysis, and experimentally tested with KASPar assays as part of the computational pipeline evaluation process for identifying putative SNPs between cotton (*G. hirsutum*) and several related 52- and 26-chromosome species (Hulse et al. *in preparation*). SNP G1_072641 can be detected by KASPar assay using the common reverse primer TGTGGAGGCATAGTGAGAGG, and forward SNP-specific primers GGTGTATGTAAAAGTCCGAAAGCA and GTGTATGTAAAAGTCCGAAAGCG. SNP Gb_010283 can be detected by KASPar assay using the common reverse primer AGATTGACTCGGGACTTCCT, and forward SNP-specific primers CCCCTCATGTTTCTAACTATTTGT and CCCTCATGTTTCTAACTATTTGC.

PCR conditions

Two μL of DNA extract were added to each well, the plate was briefly centrifuged, and dried down in a bench-top oven for 1 hour at 65°C . An 8 μL PCR mixture containing 4.0 μL of Reaction Mix (KASP Master mix, LGC Genomics #KBS-1016-002), 3.826 μL of sterile deionized water, 0.11 μL of Assay (Primer) Mix, and 0.064 μL of 50 mM MgCl_2 were added to each well. The PCR was conducted by the KASPar assays (KBioscience Ltd., Hoddesdon, UK), following an acclimation step of 94°C for 15 minutes, the first 10 cycles consisted of: denaturation at 94°C for 20 seconds, followed by annealing starting

at 65°C for 1 minute, decreasing 0.8°C per cycle to an annealing temperature of 57°C for the final cycle. This was followed by 28 cycles of denaturation at 94°C for 20 seconds, and annealing at 57°C for 1 minute. Plates were then briefly centrifuged, then read on the Pherastar plate reader. The Pherastar files were imported into KlusterCaller software for genotyping. In the event that greater amplification was required, plates were returned to the hydrocycler for iterations of 3 additional PCR cycles, with the denaturation steps at 94°C for 20 seconds, and annealing at 57°C for 1 minute.

SNP amplification for cotton seed DNA

The protocols above for non-destructive extraction of DNA from cotton seed and SNP genotyping were tested with experimental research materials. DNA samples extracted in the prescribed manner from 88 BC1F1 cotton seeds were placed into the first 11 columns of a 96-well plate, where these 88 seeds were known on the basis of pedigree to be segregating for SNP G1_072641, which is in an alien chromosome segment derived from the species *G. longicalyx*, a wild African relative of cultivated cotton. The 12th column of the same 96-well plate was dedicated to conventionally extracted DNAs from the parental leaf tissue, using Qiagen kits, and two non-template controls. All samples in the plate were amplified using the primer set for SNP G1_072641.

SNP amplification for cotton cotyledon DNA

The protocols above for non-destructive extraction of DNA from cotton seedling cotyledons and SNP genotyping were tested with experimental research materials. Using

the procedures described above, DNA was extracted from 36 BC2F1 hybrid seedlings from a *G. hirsutum**2/*G. barbadense* plant known to be heterozygous for the alien segment and were amplified using the SNP Gb_010283. DNA was extracted from young leaves of the parents using commercial kits (Qiagen). The test plate included two non-template controls and 16 empty wells.

Germination of drilled seeds after PCR analysis

Drilled seeds were stored in modified plates in cabinet drawers at room temperature of a conventional air-conditioned office (**Fig. 3.1C**). Germination rates were assessed by placing seed in two types of "ragdolls" of germination paper (Anchor Paper Co.), one pre-soaked with tap water, and other with tap water plus Benlate (0.5 ml / 100 ml tap water). Ragdolls were incubated in the dark at 30°C for 48 hours before assessment.

Results and Discussion

Non-destructive seed DNA genotyping

DNA samples extracted from BC1F1 cotton seeds using the non-destructive protocol and from leaf tissue using conventional kits were amplified with SNP Gl_072641. KASP ratios from all of the seed DNA samples fell into two well-defined clusters (**Fig. 3.3**). The patterns and positions of the clusters of seed-based SNPs were congruent with expectations for a marker that is heterozygous in the F1 hybrid parent, symbolized as (FADD) and homozygous (AADD) in the other parent. Each seed-based data point fell along the same ray (angle) as the respective control samples that had the same genotype.

The observed ratio in this batch of 88 BC1F1 seed was 44:44, which adheres exactly to the conventionally expected testcross ratio (1:1). Relative to conventionally prepared DNA from segregating seedlings, we noted that the clusters were significantly more dispersed *along* the ray (angle), which indicated more variation in overall fluorescence amplitude. We typically observed similar levels of dispersion *at right angles* to the ray, indicating that SNP-specific fluorochrome ratios were similar for the non-destructive seed-based extractions and conventional leaf-based extractions. There would seem to be two possible explanations for the additional variation in overall SNP signal amplitude, one being increased variation for DNA concentration in seed-based extractions, and the other being variation for compounds that interfere with the PCR process, such as phenolics. Regardless, several extra PCR cycles were needed for DNA samples extracted by seeds compared with conventional leaf-based extractions. The average non-destructive seed DNA extraction method provided sufficient DNA for 1000 PCR reactions; prior to PCR, the extract was diluted 20X.

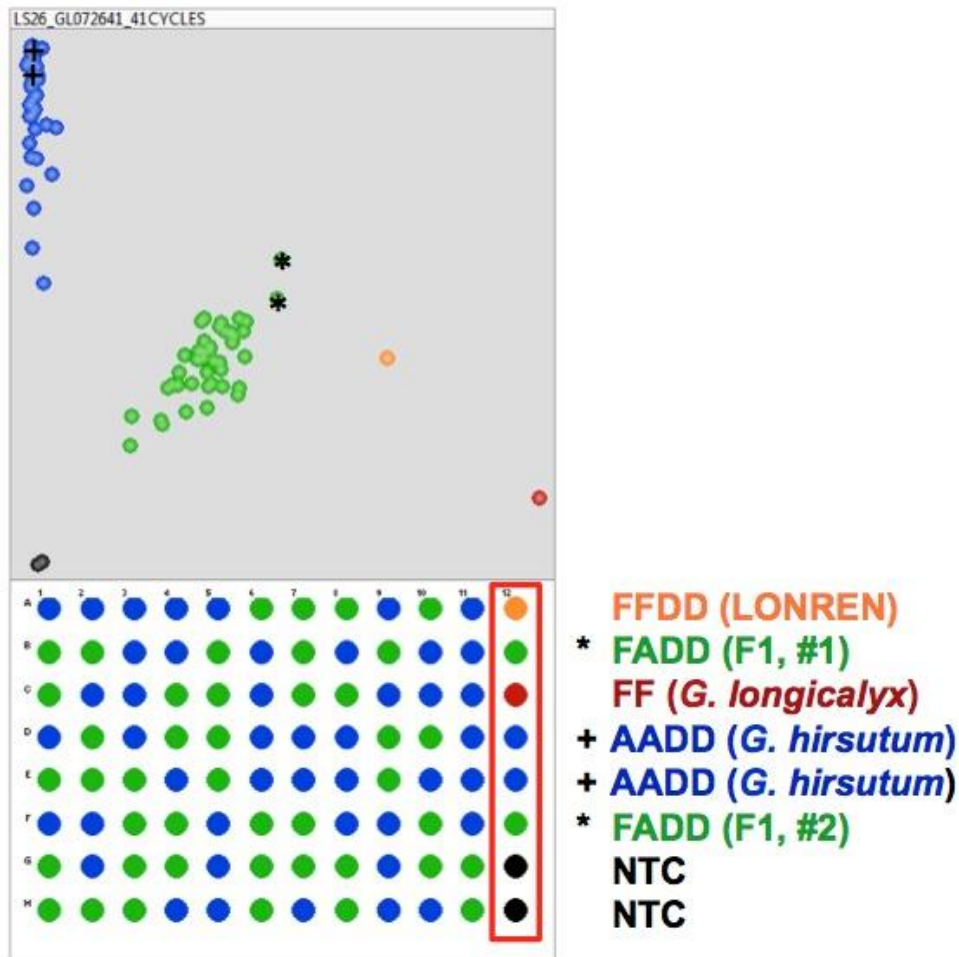


Figure 3.3. SNP genotyping of DNA extracted from individual cotton seed after 41 PCR cycles. The red rectangle denotes wells that contained DNA samples extracted using a commercial kit and fresh leaf tissue. In the two clusters, wells containing conventionally extracted DNA samples are denoted by symbols * and +. Wells containing non-template controls (no sample DNA) are black. The remaining 88 DNA samples were extracted by the non-destructive seed DNA extraction method from a backcross family segregating for SNP GI_072641.

Cotyledon DNA genotyping

DNA samples extracted from cotyledons were run in duplicate wells and amplified with SNP Gb_010283. KASP ratios from all of the seed DNA samples fell into two well-

defined clusters (**Fig. 3.4**). The patterns and positions of the clusters of seed-based SNPs were congruent with expectations for a marker that is heterozygous in the F1 hybrid parent, and homozygous in the other parent. Each seed-based data point fell along the same ray (angle) as the respective control samples that had the same genotype. The observed ratio in this batch of 36 BC1F1 seed was 19:17, which was not different from the expected testcross ratio (1:1). Relative to conventionally prepared DNA from segregating seedlings, we noted that the clusters were similarly clustered along the ray (angle), which indicates similar variation in overall fluorescence amplitude. We also observed similar levels of dispersion at right angles to the ray, indicating that SNP-specific fluorochrome ratios were similar for the cotyledon-based extractions and conventional leaf-based extractions. The cotyledon-based extractions were less variable than the non-destructive seed-based extractions. On average, the cotyledon-based extractions yielded enough DNA for 500 PCR reactions, based on 20 ng per reaction. The reduced DNA yield is likely related to the relative number of nuclei sampled. Casual observations indicated a two-fold or three-fold difference in the amount of tissue removed from cotyledons by the two extraction methods. When determining PCR conditions for the DNA extracts from expanded cotyledons, we observed superior results by limiting the pre-PCR dilution to ~10X, whereas it was ~20X for seed DNA extract. As observed for the seed-extracted DNA samples, the cotyledon- extracted DNA samples needed a few more PCR cycles than the conventional leaf- extracted DNA samples.

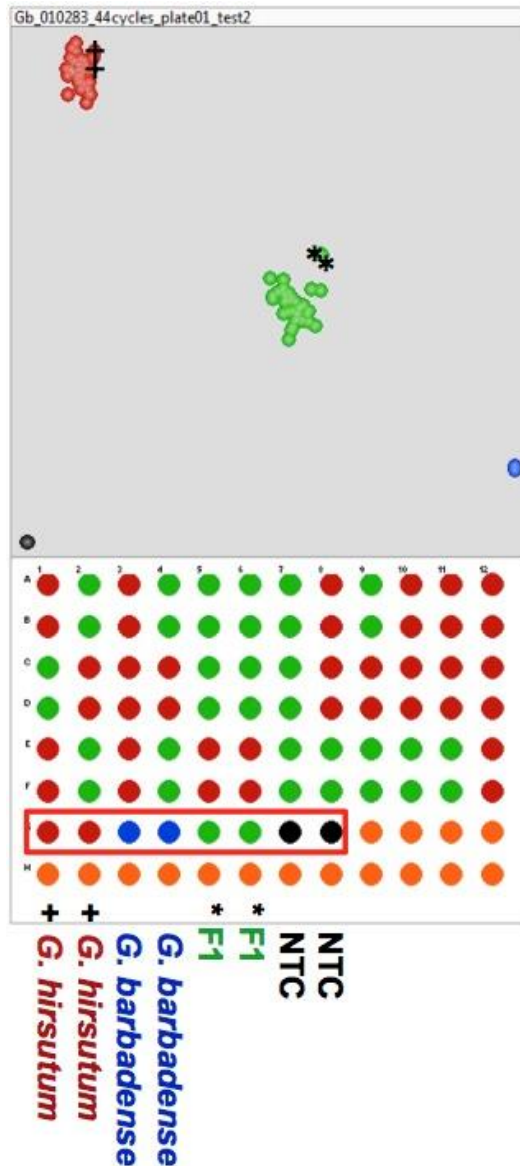


Figure 3.4. SNP genotyping of DNA extracted from individual cotton seedling cotyledons. The red rectangle denotes wells that contained DNA samples extracted using a commercial kit and fresh leaf tissue. In the two clusters, wells containing conventionally extracted DNA samples are denoted by symbols * and +. Wells containing non-template controls (no sample DNA) are black, and empty wells are colored orange. The upper 72 wells contain 36 duplicated samples from the seedling-based DNA extraction method, following 44 PCR cycles. The 36 seedlings were part of a backcross family segregating for SNP Gb_010283.

Effect of drying on cotyledon DNA extraction

Initial tests of the extraction procedures for cotyledons were based on fresh tissue, but were observed to yield inconsistent and generally undesirable results (not shown).

Given that the fresh tissue contains much more water than the non-imbibed seed, we added steps for drying and crushing the cotyledonary tissue before adding the extraction.

Good results were obtained using DNA extracted from cotyledon tissue that was dried and crushed (**Fig.3.4**).

Germination of seeds after DNA extraction

Breeders and other researchers will benefit greatly if they can genotype seed and later germinate seed that are selected based on the genotypic results. It is thus very important to know if seed would be able to germinate consistently after having their seed coats violated and sufficient amounts of tissue removed for successful DNA extractions. At the outset of experiments to develop a sampling protocol that would not significantly impair germination ability, we tested the effects of drilling slightly into 100 seed for tissue sampling, and then assessed the germination ability using conventional indoor germination procedures. All but two of the 100 seed germinated (**Fig. 3.5a**), and upon inspection we noted that those two seed had been drilled in the wrong position (near the radical). Overall, germination was excellent in laboratory and greenhouse conditions; seedlings were vigorous and no ill effects were observed except for the absence of marginal tissue (**Fig. 3.5b**). In subsequent "rag-doll" germination tests, non-drilled seed with and without fungicide treatment had nearly perfect germination percentages, as did

drilled seed with fungicide treatment (98%), whereas germination rates were slightly reduced without fungicide treatment (~90%). The germination of drilled seed in natural soils has not been tested.

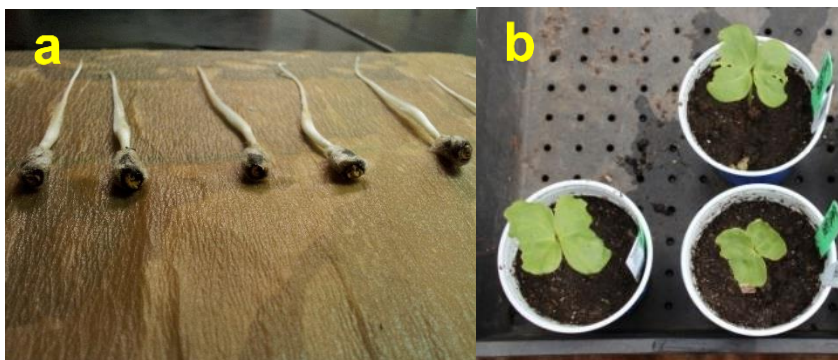


Figure 3.5. Seed germination test after non-destructive tissue sampling for DNA extraction. (a) Image of tissue-sampled seeds after germination in a "ragdoll". (b) Image of seedlings from sampled seeds, growing in soil cups; cotyledons lack areas of marginal tissue, due to previous sampling from the seed before germination.

Procedural details for seed DNA extractions

In preparing each cotton seed for tissue sampling, part of the seed coat, which is maternal not zygotic tissue, was removed by sanding. The removal provides direct access to embryo tissue, which differs from some previous seed DNA extraction methods (35). The drill bit used to sample seed tissue was cleaned between each seed by drilling into a pencil eraser, and this seemed to preclude significant cross contamination (Fig.3.1e). A modified 96-well plate was a useful holder for seed during the tissue sampling process. Holding seed by hand, tweezers and most other devices was cumbersome and inefficient, so this plate-based seed tissue sampling was more efficient

compared to single-seed handling on a flat, concave or indented surface (152). As reported previously, microtiter plates are convenient for storage of suitably small seeds (35, 152). By using the same modified plate to hold seed during sampling and storage, we avoided the need to transfer seed and minimized chances for loss of seed identity. Undelinted cotton seeds will fit more snugly in the plate, provided they are not too large. Large seed and seed size variation can cause some problems, but can be addressed by applying extra pressure to force a larger seed into a well, or applying water-soluble glue to the back of the plate. DNA yields varied somewhat from seed to seed, but it was sufficiently consistent to use for large-scale PCR based genotypic screening. It is likely that most variation in DNA yields arose from differences in the amounts of tissue obtained per seed and the relative particle size distributions, where finer particles would expectedly yield more DNA. It is also possible that differences in seed composition affected DNA extraction efficacy.

Procedure details for cotyledon DNA extractions

As noted above, a drying step was added to the process for cotyledon DNA extraction. It was observed that after drying samples with desiccant beads, the PCR seemed to be slightly more effective than after drying using an oven, possibly due to damage to DNA of fresh tissue by higher temperatures. The SNP amplification rate of cotyledon DNA was 87.5% for 136 samples dried with an oven and 100% for 60 samples dried with desiccant beads.

Reliability and scalability

This tissue sampling process for cotton seeds based on modified 96-well plates was originally developed by members of the Stelly laboratory. It has been used on over 20,000 seeds (finish in 3 weeks including extraction and genotyping by one person) and the success rate were over 95% (Chapter V). Five recombinants were verified after screening 20,000 seeds. The 5 recombinants were further confirmed by growing the seeds and extracting leaf DNA using a commercial kit (Chapter V). This 96-well plate based method for DNA extraction from cotton seeds and cotyledons, which can be directly used in KASPar assays for high-throughput genotyping, has been developed by modifying existing DNA extraction methods. Overall, a modified and optimized existing protocol was utilized, which enabled efficient / effective screening of populations (prior to planting) using SNP markers. This high-throughput DNA extraction method is cost effective (less than \$0.05/sample), non-destructive (drilled seeds exhibit high germination rates) and fast (20,000 seeds genotyped in 3 weeks by one person). The major advantage of this method is that extraction is performed on cotton seed or cotyledon tissue instead of leaf tissue, which allows MAS to be carried out before growing or transplanting. DNA from both seed and cotyledon extracts were tested and found to be amplifiable more than one year after extraction, and amenable to other PCR reactions, e.g., amplification with SSR primers (**Fig. 3.6**). Thus, the user neither needs to germinate the seed quickly, nor analyze the DNA quickly.

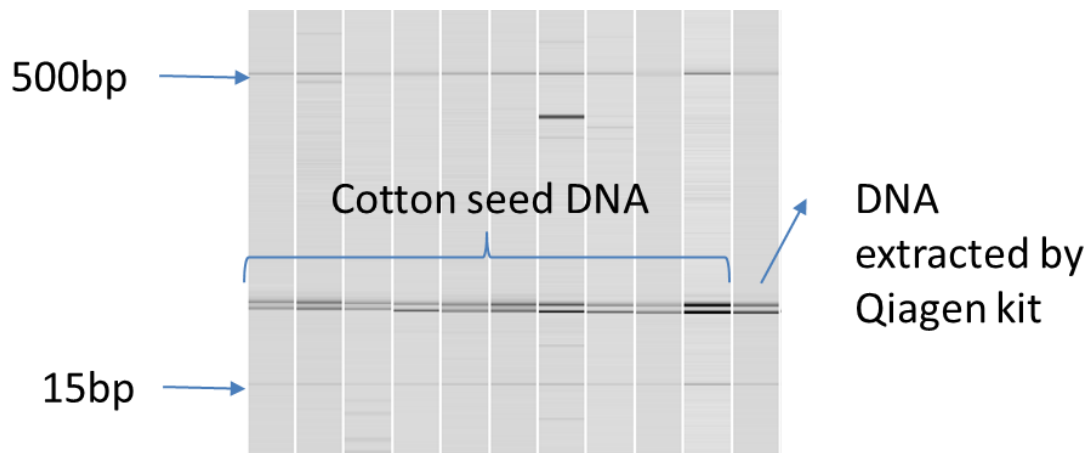


Figure 3.6. SSR genotyping of DNA extracted from individual cotton seed one year after extraction. The last lane contained DNA extracted using a commercial kit from fresh leaf tissue. The other 10 lanes contain DNA extracted by the non-destructive seed DNA extraction method from a backcross family. PCR was run using primers for SSR marker CIR 316.

Comparison to commercial kits

There is an increasing number of commercial DNA extraction products, which can vary considerably in price and applicability to various plants (173). Even though many provide high quality DNA, the cost of those commercial kits and the amount of time that they require are generally prohibitive for large sample numbers, especially for most academic plant research laboratories. There are several recent papers on rapid and cost-effective DNA extraction methods, and most of them are based on plant tissue, not seed (17, 50, 60, 86). A few DNA extractions methods have been published based on the use of pre-germinated seeds, such as barley, wheat and rice (35, 75, 152). None of them have provided a high-throughput method for cotton seeds, which is a major crop worldwide with a rapidly expanding need for marker-assisted genotyping. Since special

equipment is not needed for the DNA extractions, it will allow virtually any laboratory capable of running PCR-based markers the possibility to use genomic tools for research and breeding applications. For applications that rely on PCR, the methods described here should help meet those needs. Low-replication tests suggest the methods can be extended to a number of other plants, including crops with medium or large seed.

CHAPTER IV
DEVELOPMENT AND HIGH-RESOLUTION MAPPING OF *Ren^{lon}*-LINKED SNP
MARKERS

Introduction

The reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) (58, 87) is an increasing problem in cotton (*Gossypium hirsutum* L.) production in the eastern half of the United States Cotton Belt. It is estimated to result in annual losses of ~\$130M, with major impact in the states of Mississippi, Louisiana, and Alabama (22, 84, 124). Control of reniform nematode has been largely limited to crop rotation and application of nematicides (144). The growing problem of reniform nematode in cotton production has led to the desire to incorporate genetic resistance to the reniform nematode in Upland cotton genotypes.

Extensive screening of the *G. hirsutum* germplasm collection over the years has confirmed that Upland cotton *Gossypium hirsutum* ($2n=52, 2[AD]_1, \sim 2.5$ Gbp) germplasm lacks significant resistance to the reniform nematode (72, 125, 146, 170).

Some of the wild diploid *Gossypium* species are highly resistant to the reniform nematode, but introgression of the trait into Upland cotton is extremely difficult due to differences in their ploidy level between wild and cultivated cotton species (84).

Introgression of traits from wild species is difficult to complete and utilization in applied breeding programs is complicated and often limited by close linkage between the desired

alien gene(s) and nearby alien genes that are agriculturally undesirable. The survival of the plants resulting from many interspecific crosses is inescapably low due to chromosome pairing difficulties (9, 10) and the probability of obtaining agronomically suitable introgressed material is even lower.

In an extensive survey of *Gossypium* germplasm for reniform nematode resistance and tolerance, Yik and Birchfield (1984) found *G. longicalyx* J.B. Hutch. & B.J.S. Lee, to be immune to reniform nematode infection, whereas *G. somalense* (Gürke) J.B. Hutch. and *G. stocksii* Mast. were highly resistant. Resistance was also found in *G. arboreum* L., *G. herbaceum* L. and *G. thurberi* Tod. accessions (170). The most information regarding resistance to the reniform nematode and its introgression to *G. hirsutum* is available for *G. aridum*, *G. arboreum*, *G. barbadense* and *G. longicalyx* (122).

Romano et al. (2009), using progeny derived from a cross between a tri-species hybrid [*G. arboreum* x (*G. hirsutum* x *G. aridum*)²] and MD51ne (*G. hirsutum*), showed that resistance among introgression products was associated with the SSR markers BNL3279_132 and BNL2662_090 on chromosome 21 and originally designated the alien c21 locus as *Ren^{ari}*, as it was donated from *G. aridum* (129); the locus was later renamed *Ren₂^{ari}* (54).

An AFLP marker linked to reniform nematode resistance from *G. arboreum* was identified and tested in the BC2F1 generation (6). This resulted in a good correlation of

marker presence and resistance. However, in the BC2F2 generation the marker was not highly correlated with the resistance gene. New markers linked to resistance have been found, and their usefulness for marker-assisted selection is currently being evaluated (6).

GB-713 is the most resistant of *G. barbadense* and *G. hirsutum* accessions noted to date (123). Robinson initiated attempts to combine resistance to reniform nematode with resistance to root-knot nematode (*M. incognita*) by crossing GB 713 with either Acala NemX or M 315 RNR. A bulk-segregant analysis indicated the resistance to reniform nematode in GB 713 was controlled by a single dominant gene with additive effects (125). Based on segregation and linkage analyses from progenies involving hybrids with Acala Nem-X, resistance of GB713 was attributed to three resistance genes, including two in chromosome 21, and one in chromosome 18 (61). One QTL on chromosome 21 was at map position 168.6 (LOD 28.0) flanked by SSR markers, BNL1551_162 and GH 132_199 at positions 154.2 and 177.3, respectively. A second QTL on chromosome 21 was at map position 182.7 (LOD 24.6) flanked by SSR markers BNL4011_155 and BNL 3279_106 at positions 180.6 and 184.5, respectively. One QTL with smaller genetic effects was localized to chromosome 18 at map position 39.6 (LOD 4.0) and flanked by SSR markers BNL 1721_178 and BNL 569_131 at positions 27.6 and 42.9, respectively (61).

The African wild species, *G. longicalyx* ($2n=26$, $2F_1$, ~ 1.3 Gbp) was found to be the most reniform-resistant *Gossypium* species, and deemed completely resistant (170).

Transfer of reniform nematode resistance from *G. longicalyx* (F1) to Upland cotton lines was achieved by [1] “Tri-species hybrid synthesis [$(G. hirsutum / G. longicalyx) 2n=6x // G. armourianum$ (designated "HLA" tri-species hybrid) and $(G. hirsutum / G. herbaceum) 2n=6x // G. longicalyx$ (designated "HHL")], [2] modified backcrossing (BC), and [3] inbreeding. A gene conferring “immunity” to reniform nematodes was thereby transferred from the wild species *Gossypium longicalyx* by homologous recombination with Upland cotton chromosome 11, and two of the resulting reniform-resistant upland lines were released for more extensive evaluation and breeding in 2007, namely LONREN-1 and LONREN-2 (48, 124). Microsatellite (SSR) markers were developed to tag this gene, and the closest marker BNL3279_114 was found to reside < 1 cM away from the gene (48). The linkages led to extensive use of marker-assisted selection (MAS) in cotton breeding programs for resistant and susceptible types, as MAS was more effective, less time-consuming and less expensive than high quality phenotypic screening. Broader evaluations of the LONREN lines and derived materials quickly revealed that plants bearing the alien *Ren^{lon}* segment from *G. longicalyx* were debilitated when grown in naturally nematode-infested fields. LONREN and derived resistant lines were smaller and less productive on average than isogenic susceptible lines in spite of their resistance to the nematodes (15).

The list of potentially contributing factors to stunting has included root damage caused by hypersensitivity to the nematode, per se, and/or new or accentuated susceptibility to one or more soil-borne pathogens, e.g., due to loss of linked (repulsion) *G. hirsutum*

disease resistance loci (174). Several nematode and fungal resistance loci of cotton are known to reside nearby. It seems unlikely but is certain possible that the gene for nematode resistance directly affects multiple traits, rather indirect effects seem much more likely, e.g., due to tissue damage from hypersensitive cell death. Additionally, closely linked genes would be expected to affect multiple traits, including pathogen resistance and agronomic performance and quality. Such effects could be due to direct effects of specific genes on specific pathways and traits, while others might be quite indirect. Of special interest may be other genes that affect resistances to other pathogens, because many genes important to resistances are known to be clustered and so it would be quite likely that the gene for reniform resistance is flanked by other *G. longicalyx* genes that alter the profile of resistances to multiple pathogens.

In any case, high density of *Ren^{lon}*-linked markers are needed in order to better characterize the *Ren^{lon}* flanking region. Several recently mapped SSR markers from public resources and maps were found to be linked with *Ren^{lon}*. We also discovered 85 *Ren^{lon}*-linked SNPs. Such efforts were facilitated by the availability of the D5 genome assembly, which was released in early 2012 (111), and the availability of large numbers of putative SNPs between *G. longicalyx* and *G. hirsutum* (*Gh_Gl* SNPs) deduced by RNA-seq (Chapter II). Using the D5 genome assembly as a reference for "mapping" (sequence alignment by BLASTN analysis), hundreds of *Gh_Gl* SNPs were localized in the *Ren^{lon}* region. The putative *Gh_Gl* SNPs mapped in the *Ren^{lon}* region according to the D5 genome were tested against a screening panel that confirmed them as

experimentally useful SNPs and localized them to the region of interest. Validated *Gh_Gl* SNPs were selected and used for high-resolution mapping. Eight SNPs on each side of *Ren^{lon}* were selected and mapped on 1760 individuals from two different testcross families, PCOs and DCOs. Due to the low homeologous recombination rate between *G. hirsutum* and *G. longicalyx*, we are unable to separate all the SNPs.

Materials and Methods

Plant materials

Plant materials included tri-species hybrid consisting of *G. hirsutum* (AD₁) as the recipient species, *G. longicalyx* (F₁) as the donor parent, and a wild diploid, *G. armourianum* (D₂₋₁). The hybrids were designated by the initials HLA for [(*G. hirsutum* x *G. longicalyx*) chromosome-doubled x *G. armourianum*]. The developmental details of the tri-species hybrids are summarized by Bell and Robinson (2004) (13). Two sister germplasm lines LONREN-1 and LONREN-2 containing the introgressed *G. longicalyx* gene *Ren^{lon}* were jointly released by the USDA-ARS, Texas A&M AgriLife Research and Cotton Incorporated (14). In addition to the two released LONREN lines, more lines containing *Ren^{lon}* were developed, each differing in pedigree and/or alien segment constitution. The lines were categorized according to recombination events relative to *Ren^{lon}*, the centromere, mapped SSR markers, the phenotypic marker *Fzg^{lon}* and the telomere (48). The *Ren^{lon}*-containing introgression lines were classified into one of two basic types, DCO and PCO (**Fig. 4.1**).

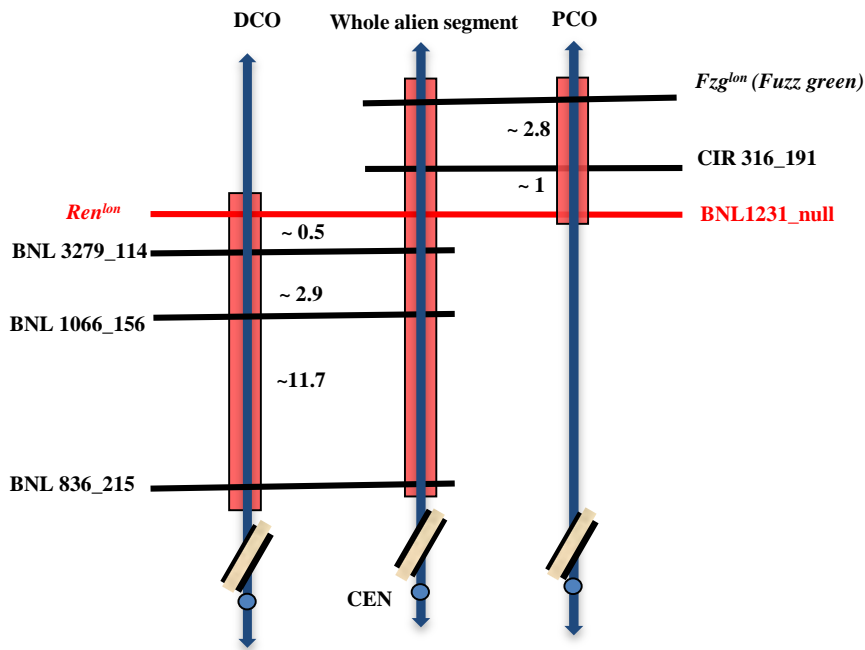


Figure 4.1. Diagrammatic representation and categorization of DCO and PCO recombination products characterized with the aid of SSR markers that closely flank the *Ren^{lon}* gene. Prior to recombination between these SSRs, the alien segment (red) is large and spans both SSRs. A nearby crossover can be detected by loss of an alien marker. Nearby distal crossovers (DCOs) lack CIR 316_191 (right) and nearby proximal crossovers (PCOs) lack BNL 3279_114 (left).

"DCO" resistance lines carry the product of a previous crossover distal to the *Ren^{lon}* gene, i.e., they contain a proximal alien segment which can be identified by SSR markers BNL3279_114, BNL1066_156 and BNL836_215. Each "PCO" resistance line carries the product of a proximal crossover (between *Ren^{lon}* and the centromere), and contains the distal alien segment which can be identified by SSR marker CIR316_191 and phenotypic marker *Fzg^{lon}*.

The two classes are exemplified by advanced backcross lines 7117(PCO) and 7123(DCO), both of which exhibit high resistance to reniform nematode. The pedigrees of 7117 and 7123 are shown in **Table 4.1**. Backcrosses were used to create a large BC1F1 populations of both lines for high-resolution mapping. Seeds of both homozygous lines were acquired from A. A Bell, (USDA-ARS at College Station, TX), and planted in the green house over the fall of 2009 at College Station, TX. Crosses were made with *G. hirsutum* ‘FiberMax 966’ (FM 966) to create *Ren^{lon}* heterozygotes in the early spring of 2009. F1 seeds were collected, sorted and ginned. F1 plants were backcrossed (testcrossed) to FM 966 to construct the large BC1F1 populations in the summers of 2010 and 2011 in the field in College Station. A set of pollination-specific seed envelopes was randomly selected from the 2011 summer backcrosses for high-resolution mapping, providing a sample of 880 BC1F1 seeds.

Table 4.1. Pedigree of 7117 and 7123.

Plant ID	Resistance Source*	Recombination type	Parents/ Generation					
			BC2	BC3	BC4	BC5	BC6	BC7
7117	HLA-B45	PCO	DPL 458	DPL 458	FM 958	DPL 5415	STV 474	NemX
7123	HLA-A85	DCO	Nemx	DPL 458	STV 454	FM 958	STV 474	NemX
* HLA-B45 group BC1: pollen from Acala NemX HLA-A85 group BC1: a pool of <i>G. hirsutum</i> pollen from SG125, DP373, PM1220, TAMCOT Sphinx								

DNA extraction

DNA samples of the screening panel and parental controls from each mapping population were extracted using young, folded or newly unfolded leaves by Qiagen

DNeasy Plant Mini Kit according to the manufacturer's protocol. DNA yield was estimated using a NanoDrop2000 and the DNA diluted to 10ng/ μ l for PCR amplification.

DNA samples from the progeny of each mapping population were extracted from undelinted seeds using the seed DNA extraction method described in Chapter III. DNA samples extracted from 88 seeds were in each 96-well plate with the last plate column containing parental control DNA and non-template control. The DNA samples were diluted 20X.

Ren^{lon}-linked SNP markers

By previous efforts, a total of 106,795 *Gh_Gl* SNPs were developed by RNA-seq and *in silico* filters and comparisons as described in Chapter II. The estimated rate of conversion from *in silico* SNPs to usable SNP markers (KASPar assays) for the whole F1 (*G. longicalyx*) genome was over 90% (Chapter II). The RNA-seq derived *Gh_Gl* SNPs and previously published SSR markers on the *Ren^{lon}* map (48) were aligned to the *G. raimondii* D5 genome sequence assemblies, which were released in early 2012 (<http://www.phytozome.net/cotton.php>) (111), using the BWA program with default settings. The SNPs which mapped in the *Ren^{lon}* region were selected for validation with a screening panel. There are three classes of *Gh_Gl* SNPs: Class I (Clean SNPs – No intron - No INDEL – No GSP in the SNP-containing contig), Class II (No intron-No INDEL – No GSP within 100 bases of the SNP), and Class III (No intron within 100

bases of the SNP but One GSP/INDEL within 100 bases of the SNP). Only Class-I and Class-II *Gh_Gl* SNPs mapped in the *Ren^{lon}* region were selected and used for validation. Allele-specific primers and allele-flanking primers for the selected SNPs were designed using the Batch primer3 software program. Default settings were used to select both the SNP-specific and flanking primers with the only exception being maximum product size of 100bp, minimum product size of 50bp; minimum primer T_m 55°C, and maximum primer T_m 63°C. Likewise, default settings were also used for penalty weights. Desirable SNP markers were validated against the screening panel by KASPar assays (KBioscience Ltd., Hoddesdon, UK). A 6x4 format with 2 replicates of each genotype were used for screening *Ren^{lon}* linked SNPs (**Table 4.2**). Plates were originally run for the optimum 38 cycles on the LGC genomics SNP line, centrifuged then read on the Pherastar plate reader. The Pherastar files were imported into KlusterCaller software for genotyping. If the plates were not acceptably clustered and did not have scoreable genotypes, an additional set of 3 cycles was added and the plates were read and imported again, this was repeated until scoreable clusters were formed.

The screening panel consisted of parental controls and several different homozygous and heterozygous reniform nematode resistance lines (**Table 4.2**). ‘FM 966’ and ‘Acala Nemx’ (Nemx) were used as susceptible controls, and they are also backcross parents used for the advanced reniform nematode resistant lines. *G. amourianum* was used as a bridge when creating the original HLA tri-species hybrid. *G. longicalyx* provided the reniform nematode resistance gene, *Ren^{lon}*.

Table 4.2. Screening panel for *Ren^{lon}* linked SNP markers.

7127	7123	7117	7131	3401	3402
7127	7123	7117	7131	3401	3402
HLA	G. lon	FM 966	G. arm	Nemx	HTC
HLA	G. lon	FM 966	G. arm	Nemx	HTC

7127, 7123, 7117, 7131, 3401 and 3402 are the different nematode resistance lines, and were derived from the original HLA family.

7127 & 7123 bear different distal cross over (CO) events.

7117 bears a proximal CO.

7131 bears a distal CO (heterozygous).

3401 & 3402 contain large alien segments, where 3401 is heterozygous.

HLA is the tri-species hybrid. *G. longicalyx*, FM 966, *G. armourianum*, Nemx are the parents of HLA or the backcross parents of these different resistance lines.

High-resolution mapping

The SNPs used for high-resolution mapping were selected from the validated *Ren^{lon}*-linked *Gh_Gl* SNPs. The recombination rate between *G. longicalyx* and *G. hirsutum* is relatively low, so the selected *Gh_Gl* SNPs were equally distributed with some distances according to the D_5 alignment results.

SNP amplification was performed in 96-well plates using the KASPar assay as previously described. Genotype files were uploaded to MSTmap online, with single linkage group (LG) for Grouping LOD Criteria. Default settings (<http://138.23.178.42/mstmap/>) were used to estimate the genetic distance of the mapped SNP markers.

Results

Ren^{lon}-linked SNPs

By aligning mapped SSR markers with *Ren^{lon}* to the D5 genome assembly, Scaffold_7 (Chromosome_7) was identified to be the region of interest (**Table 4.3**). Hundreds of *Gh-Gl* SNPs (Class I and Class II) were aligned to this D₅ region of interest. We eliminated SNPs that were repetitive, SNPs from the same RNA-seq contig, and lastly, SNPs for which good KASP primers could not be designed. Finally, 126 *Gh-Gl* SNPs from the region of interest were selected and primers designed. After testing against the screening panel (**Table 4.2**) using the KASPar assay, 86 *Gh-Gl* SNPs were validated with linkage to the *Ren^{lon}* gene (SNP sequences and primers are in **additional file 2**). Markers were distributed along a relatively large D5 segment that aligned to the targeted region.

Table 4.3. Mapped SSR markers aligned to the D5 assembly.

Mapped SSR markers	Scaffold or Chromosome	Position on D5_V2.1
CIR316	07	59563000
BNL1231	07	57124927
BNL3279	07	56142974
BNL1066	07	54550465
BNL836	07	52098818

The SSR markers in **Table 4.3** are from the genetic map of Dighe et al. 2009 (48). All of them aligned to D5 Chr. 07 by using BWA with default settings. The position on D5 is either by the primer start position or the marker sequence.

High-resolution mapping

Two *Gh_Gl* SNPs were selected in the common region of PCOs and DCOs. The two SNPs aligned on both sides of SSR marker BNL1231 based on the D5 alignment results. Eight SNPs were selected for both proximal and distal regions, and they were roughly equally distributed across the region. Selected *Gh_Gl* SNP sequences and primers are highlighted in **additional file 2**.

The genetic order of the 10 *Gh_Gl* SNPs for the 7123 family was the same as the order obtained following alignment to the D5 genome (**Table 4.4**). Seven recombination events were found in the 7123 (DCO) family. The recombinants were confirmed twice by SNP markers. The alien segment of 7123 was located between SSR markers BNL1231 and BNL836. The mapping results were confirmed using *Ren^{lon}* linked SSR markers.

No recombination events were found in the 7117 (PCO) family (**Table 4.4**), although 8 *Gh_Gl* SNPs were selected in this region plus 2 *Gh_Gl* SNPs in the common region of the DCOs and PCOs and they were distributed from SSR marker BNL1231 to

phenotypic marker *Fzg^{lon}*, which is thought to be located very near the end of the chromosome-11 in *G. hirsutum*.

Table 4.4. High-resolution mapping results of *Ren^{lon}*-linked SNPs.

SNPs for DCO	Position on D5_V2.1	MST MAP POSITION (cM)
GI_178356	52275364	0
GI_117394	52513762	0
GI_085251	52847958	0.452
GI_200380	53688922	0.931
GI_187401	54605631	1.341
GI_142101	55188861	1.674
GI_012150	55404836	1.674
GI_117570	55971263	1.674
GI_168758	56835707	2.174
GI_072641	57279176	2.610
SNPs for PCO	Position on D5_V2.1	MST MAP POSITION (cM)
GI_168758	56835707	0
GI_072641	57279176	0
GI_212476	57435262	0
GI_052088	57775963	0
GI_148225	57834425	0
GI_104307	58465267	0
GI_211386	58627706	0
GI_199350	59030131	0
GI_208281	59481497	0
GI_082005	60340986	0

The 7 new recombinants found in the 7123 family were confirmed with 10 relevant *Gh_Gl* SNP markers (**Table 4.5**). The seeds were selfed and the resulting seeds collected for use in future germplasm evaluation.

Table 4.5. New recombinants identified from BC1F1 progeny from family 7123.

7123 family	Gl_178 356	Gl_117 394	Gl_085 251	Gl_200 380	Gl_187 401	Gl_142 101	Gl_012 150	Gl_117 570	Gl_168 758	Gl_072 641
D1F3	H	H	H	H	H	H	H	H	H	A
D1H4	H	H	H	H	H	A	A	A	A	A
D2C10	H	H	A	A	A	A	A	A	A	A
D3E4	H	H	H	A	A	A	A	A	A	A
D3H1	A	A	A	A	H	H	H	H	H	H
D6F4	A	A	A	A	A	A	A	A	H	H
D8A11	A	A	A	A	A	A	A	A	H	H

A: homozygous for *G. hirsutum* allele.
H: heterozygous for *G. longicalyx* allele.

Discussion

For identifying *Ren^{lon}* linked SNP markers, the D5 genome assembly was used as a reference. The *Gh_Gl* SNPs developed by RNA-seq were aligned to D5 using the BWA program, as were the SSR markers previously mapped to *Ren^{lon}* (48). Over 70% of the the *Gh_Gl* SNPs were mapped to the D5 assembly (Chapter I). The *Ren^{lon}* region was identified by the position of *Ren^{lon}* linked SSR markers aligned on D5. The desirable SNPs aligned on the region of interest were validated using KSAPar assays with a screening panel of selected cotton cultivars. In total 86 *Gh-Gl* SNPs were validated as

linked with *Ren^{lon}* across the whole alien segment on chromosome-11 from analysis of the data obtained with the screening panel. A-genome diploids native to Africa and Mexican D-genome diploids diverged ~5–10 MYA (135). Several lines of evidence indicate that *G. longicalyx* from Africa with the F-subgenome is more closely related to the A-subgenome than the D-subgenome (112). Not all the the potential SNPs of interest can be found by the alignment results of *Gh_Gl* SNPs against D5 genome assembly.

The BC₁F₁ families from 7117 and 7123 were selected for high-resolution mapping, because they are two different types of previous recombination events, PCO and DCO. Lines 7117 and 7123 contained shorter alien segments as revealed following analysis with newly developed *Ren^{lon}*-linked SSR markers. In other words, the common *Ren^{lon}* flanking region between 7117 and 7123 was smaller than other combinations of PCOs and DCOs. For each family, 880 BC₁F₁ seeds were selected for high-resolution mapping.

The seed DNA extraction method is cheap, non-destructive and fast. This method works well for large numbers of DNA extracts that are needed in a manner that is especially time and cost efficient. In total, 880 samples from each family were mapped with 10 markers. Thus the total number of data points collected was $880 \times 10 \times 2 = 17,600$. Three hundred and eighty-five (2.1875%) data points were missing, confirming the robust nature of the seed DNA extraction method developed.

The effort to achieve high-resolution mapping around *Ren^{lon}* was affected by the low rate of recombination in this chromosomal region. Only a partial high-resolution map was constructed for the DCO family containing the proximal alien segment. The mapping results revealed that no recombination events were identified in the 7117 (PCO) family, and only 7 recombinants were identified in the 7123 (DCO) family. The 10 *Gh_Gl* SNPs used in mapping the 7117 family aligned to the D5 assembly on Chr. 07 from 56835707 to 60340986bp, and the alien *Ren^{lon}* segment (from SSR marker BNL1231 to phenotypic marker *Fzg^{lon}*) is ~ 3.8 cM based on the previous genetic map made between *G. longicalyx* introgression resistance lines and *G. hirsutum* (48). For 7123 family, the 10 *Gh_Gl* SNPs used aligned to D5 Chr. 07 from 52275364 to 57279176bp corresponding to ~15.1 cM on the genetic map. The recombination rate was particularly depressed in these advanced backcross generations compared to the previous genetic map.

The high-resolution map was limited primarily by the lack of sufficient recombination events in the *Ren^{lon}* chromosomal region, which is due to the especially low recombination rate between the alien segment from *G. longicalyx* and chromosome 11 of *G. hirsutum*. However the *Gh_Gl* SNP markers developed are now available and will be useful for studying the *Ren^{lon}* region in the future. The two closest *Gh_Gl* SNP markers, *Gl_072641* and *Gl_168758*, are ~0.4 cM apart in the current map and have been used to screen the recently released LONREN germplasm (14). The introgressed segment containing *Ren^{lon}* is valuable for more than just resistance to reniform nematode, as it

also harbors greater fiber quality traits (160). All of the validated *Ren^{lon}* linked *Gh_Gl* SNP markers will be valuable in identifying new recombinants by MAS, as well as in facilitating future germplasm introgression from *G. longicalyx*. The seven new recombinants with different alien segments will be potentially useful germplasm in the future, as well.

CHAPTER V
SCREENING FOR NEMATODE-RESISTANT RECOMBINANTS WITHOUT
“STUNTING”

Introduction

A nematode resembling reniform was first reported in Georgia, US in the 1940 (138). Reniform is now recognized as a serious pest of Upland cotton (*Gossypium hirsutum* L.) and soybean (*Glycine max* L.) in the southern United States (124). It is estimated to cause annual losses of approximately \$130M, with major impacts in Mississippi, Louisiana and Alabama (22, 84, 124). Upland cotton *Gossypium hirsutum* (2n=52, 2[AD]₁, ~2.5 Gbp) cultivars lack significant resistance to reniform nematode. Even though more than 2000 *G. hirsutum* accessions have been evaluated (126, 150, 159), only weak to moderate resistance was found. The African wild species, *G. longicalyx* (2n=26, 2F₁, ~1.3 Gbp) was found to be the most resistant *Gossypium* species (170). Transfer of reniform nematode resistance from *G. longicalyx* (F1) to Upland cotton lines was achieved by the development of the tri-species hybrid, "HLA", [(*G. hirsutum* x *G. longicalyx*)²] x *G. armourianum* Kearn.], followed by backcrossing and inbreeding (13, 124). A gene conferring “immunity” to reniform nematodes was thereby transferred from the wild species *G. longicalyx* to *G. hirsutum*. Linkage with molecular and phenotypic markers indicated that a single dominant gene or haplotype *Ren^{lon}* in chromosome 11, was responsible for resistance (48). The germplasm lines LONREN-1

and LONREN-2 have this resistance gene and were jointly released by the USDA, Texas AgriLife Research and Cotton Incorporated (48, 124).

The LONREN cotton lines are virtually immune to reniform nematode (*Rotylenchulus reniformis*) in controlled inoculation studies. Yet when planted in nematode-infested fields these lines often show severe stunting at two to three weeks after planting, compared to their nematode-susceptible sibs or commercial cultivars (15). BARBREN-713 released in 2012 suppresses reniform populations by 70-90%, but does not show any stunting problem in nematode-infested fields. The resistance in GB-713, the resistant parent of BARBREN-713, was attributed to three resistance genes, *Ren^{barb1}*, *Ren^{barb2}* and *Ren^{barb3}* located in chromosome 21 and 18 (61).

Recent evidence indicates that LONREN germplasm is differentially sensitive to high-nematode populations, and suffers considerable root damage, that leads to stunting (136). Controlled experiments indicate that genotypes with *Ren^{lon}* are also differentially susceptible and/or sensitive to specific soil-borne fungi, including *Thielaviopsis basicola*, in the presence of reniform nematode(12). The increased susceptibility could be due to a hypersensitive reaction to reniform nematodes and (or) nearby alien genes that modify resistance to one or more soil fungi (“linkage drag”). Introgressing *Ren^{lon}* into c21 rather than c11 did not avoid stunting, and indicated that loss of native c11 resistance genes does not account for the stunting (16). Hybrids created between BARBREN and LONREN also show stunting, indicating that the factor(s) that lead to

stunting are dominant and (or) epistatic to the non-stunting nature of BARBREN713 resistance (16).

The list of factors potentially contributing to stunting include severe root system damage caused by hypersensitivity to the nematode, and/or increased susceptibility to soil-borne microbial pathogens. Another possibility is that replacement of cotton DNA by alien DNA inadvertently included loss of one or more allelic or linked *G. hirsutum* genes that normally provide resistance to soil-borne pathogen(s) (174). To avail growers the maximum benefits of nematode resistance, cultivars with resistance should be free of special concerns and, if possible, not require specialized weed management practices. Thus, eliminating the "stunting", if possible, is deemed to be an important practical goal.

Usually, introgression breeding carries a cost, namely, genetic linkage of non-targeted loci that are eliminated through repeated backcrossing. Linkage drag can persist within a genome despite backcrossing, especially if recombination is suppressed. Linkage drag has been considered as one of the biggest concerns in introgressing traits from unadapted germplasm into adapted germplasm. Several examples of linkage drag in tomato and other crops have been quantified using molecular markers (62, 80, 117, 151, 172).

Linkage drag can denote favorable, deleterious or neutral alleles that become inadvertently incorporated into breeding lines or cultivars.

When the source of a gene is wild germplasm, linkage drag could be difficult to remove by traditional backcross procedures. Marker analysis can help to solve the problem.

Breaking the linkage by MAS offers the simplest approach to selecting reniform-resistant cottons that are free of unwanted genetic effects of linked alien genes. Products from recombination would not be genetically modified, and would be readily bred and deployed in US grower fields.

A total of 17,600 BC1F1 progenies were screened by high-throughput non-destructive DNA extraction methods based on seeds and genotyping of the two closest *Ren^{lon}*-linked SNP markers. Five recombinant seed with minimized flanking regions were found among those 17,600 backcross seeds. Tests for stunting and reniform nematode resistance were carried out for the selfed progenies from the five recombinants to determine whether the new recombinants are resistant to reniform nematode but free of stunting. Progeny from two of those five recombinants were free of stunting. Based on tests with nematodes, the progeny from one of the two stunt-free recombinants had lost resistance to the nematode as a consequence of the crossover, while the other segregated for resistance, suggesting that it might separate the resistance and the stunting. Further tests will be needed to confirm resistance and verify loss of stunting in the resistant progeny.

Materials and Methods

Materials for large-scale screening

Eighteen different nematode-resistant homozygous lines with different previous recombination events between flanking SSR markers were used for the study. All of the lines were selected from diverse pedigrees of the advanced backcross program (**Table 5.1**). HLA-A group BC1: a pool of *G. hirsutum* pollen from SG125, DP373, PM1220, TAMCOT Sphinx; HLA-B group BC1: pollen from Acala NemX. Lines 7117, 7118 and 7119 have proximal cross overs (PCOs), and they contained a distal alien segment with SSR markers CIR316_119 and *Fzg^{lon}*. The other 15 lines (DCOs) have distal cross overs, and they contained a proximal alien segment with SSR markers BNL3279_114, BNL1066_156 and BNL1066_215 (as described in Chapter IV).

Backcrosses were used to reduce the alien segment size by identifying new recombination events near the *Ren^{lon}* gene (**Fig. 5.1**). Seeds of 18 lines putatively homozygous for previous recombination events near *Ren^{lon}* were obtained from A. A. Bell in 2009 (USDA-ARS at College Station, TX). Seeds were planted in a greenhouse in the fall of 2009, and testcrossed with *G. hirsutum* ‘Fibermax 966’ to yield *Ren^{lon}* heterozygotes (F1s) in spring 2009. Seeds were collected, sorted and ginned. F1 plants from the 18 lines were backcrossed with FM 966 in summer 2010 and summer 2011 in College Station, at the University’s F&B Road field. Backcross seeds (BC1F1) from 18 different nematode-resistant homozygous lines were randomly selected, and 17,600 were used for screening in this study.

Table 5.1. Pedigree of different parents used to obtain *Ren^{lon}* recombinants.

Plant ID	Resistance Source	Parent ID, Generation, and Marker information	Parents/Generation					
			BC2	BC3	BC4	BC5	BC6	BC7
7109	HLA-A132	MO30-1 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	Aub 623	DPL 458	DPL 458	STV 474	FM 958	NemX
7110	HLA-A122	MM9 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	NemX	DPL 458	DPL 458	STV 454	STV 454	NemX
7111	HLA-A103	ML43-2 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	DPL 458	DPL 458	DPL 458	STV 454	FM 958	NemX
7112	HLA-A103	ML42-10 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	DPL 458	DPL 458	DPL 458	STV 454	FM 958	NemX
7113	HLA-A103	ML37-12 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	DPL 458	DPL 458	DPL 458	STV 454	FM 958	NemX
7114	HLA-A103	ML24-10 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	DPL 458	DPL 458	DPL 458	STV 454	FM 958	NemX
7115	HLA-A103	ML20-5 BC7S2 =wh fz, 3279-lon, 316-Nemx, RKN-1	DPL 458	DPL 458	DPL 458	STV 454	FM 958	NemX
7116	HLA-A84	ME25-19 BC7S2 = wh fz, 3279-lon, 316-Nemx, RKN-1	DPL 458	DPL 458	DPL 458	STV 474	FM 958	NemX
7117	HLA-B45	7A-14 BC7S1 = gr fz, 3279-hir, 316-lon	DPL 458	DPL 458	FM 958	DPL 5415	STV 474	NemX
7118	HLA-A85	MF-3 BC7S1 = gr fz, 3279-hir, 316-lon	NemX	DPL 458	DPL 458	STV 474	FM 958	DPL5 415
7119	HLA-B91	12-11 BC7S1 = gr fz, 3279-hir, 316-lon	NemX	DPL 458	FM 958	DPL 5415	STV 474	NemX
7121	HLA-A103	ML-38 BC7S1 = wh fz, 3279-lon	DPL 458	DPL 458	DPL 458	STV 474	FM 958	NemX
7122	HLA-A85	MK-27 BC7S1 = wh fz, 3279-lon	CBL 413	DPL 458	STV 454	FM 958	STV 474	NemX
7123	HLA-A85	MH-8 BC7S1 = wh fz, 3279-lon	NemX	DPL 458	STV 454	FM 958	STV 474	NemX
7124	HLA-A85	MG-15 BC7S1 = wh fz, 3279-lon	NemX	DPL 458	DPL 458	STV 474	DPL 5415	NemX
7125	HLA-B26	4(NX)-4 BC7S1 = wh fz, 3279-lon	DPL 458	DPL 458	FM 958	DPL 5415	STV 474	NemX
7126	HLA-A84	MR-7 BC7S1 = wh fz, 3279-lon	NemX	DPL 458	FM 966	DPL 5415	DPL 5415	DPL 493
7127	HLA-A103	85-10 BC8S1 = wh fz, 3279-lon	DPL 458	DPL 458	DPL 458	FM 966	FM 966	FM 966
7131	HLA-A103	85-10 BC8S1=Fzg heterozygous, 3279-lon	DPL 458	DPL 458	DPL 458	FM 966	FM 966	FM 966

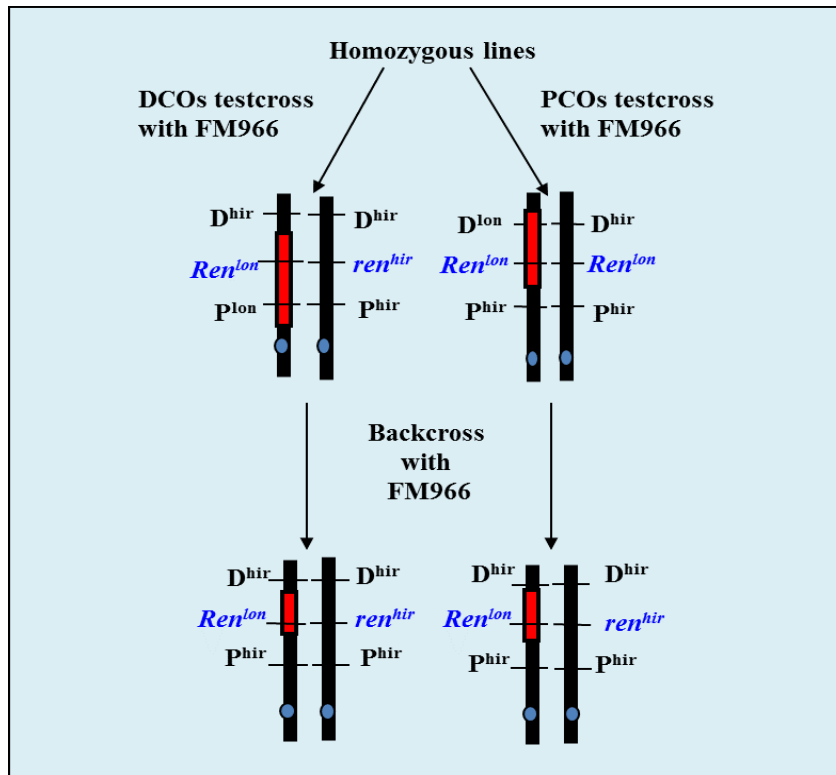


Figure 5.1. Backcross method to minimize alien segments.

DNA extraction

DNA of individuals from the backcross populations was extracted from undelinted seeds using the seed DNA extraction method (Chapter III). Seed samples were placed in 96-well PCR plates with the last column empty for parental controls and non-template control. Eighty-eight BC₁F₁ seeds or DNA samples were put in each plate. The DNA samples were diluted ~20X.

DNA of parental controls were extracted using young, folded or newly unfolded leaves using the Qiagen DNeasy Plant Mini Kit, and the DNA yield was estimated using a NanoDrop2000. DNA then was diluted to 10ng/μl for PCR amplification.

MAS for identifying best recombinants

The *Ren^{lon}*-linked *Gh_Gl* SNP markers were developed by D5 genome assembly alignments and KASPar assay validation as described in Chapter IV. The two closest SNPs, *Gl_168758* and *Gl_072641*, on opposite sides of SSR marker BNL1231 were selected and used for large-scale screening of backcross seeds to identify recombinants with breakpoints very close to *Ren^{lon}* (**Table 5.2**).

Table 5.2. Two closest *Ren^{lon}*-linked SNPs.

SNP name	Position inD5	SNP sequences	Common primer	Allele specific primer 1	Allele specific primer 2
<i>Gl_168758</i>	56835707	AGATCCTGAGATTCGAAGCCAAA ATTGACATCAACTCGAGTGCGAG CTTT[G/A]TCGATCTGTCAGACCC CTTTCTTCTTTTGCTTCTTCTTGC GCAAATAATT	TATTTG CGCAA GAAGA AGCA	AACTCG AGTGCG AGCTTTG	CAACTC GAGTGC GAGCTT TA
<i>Gl_072641</i>	57279176	GAGGCGATGAAGATCAAATCAC TGTGGAGGCATAGTGAGAGGTTA GCTAT[C/T]GCTTTCGGACTTTTA CATACACCGGCAGGAAGCTTGAT AAGGATCAGTAA	TGTGG AGGCA TAGTG AGAGG	GGTGTAT GTAAAA GTCCGA AAGCA	GTGTAT GTAAAA GTCCGA AAGCG

Two hundred 96-well plates of seed DNA samples (17,600 samples) were screened using the two SNP markers, *Gl_168758* and *Gl_072641*, which are closest to *Ren^{lon}*. The LGC Genomics/Kbioscience KASPar assay (<http://www.lgcgenomics.com/>) was used

for SNP amplification. Allele-specific and allele-flanking primers were developed using Batch Primer-3 software with an optimal primer T_m of 57°C (minimum 55°C, maximum 60°C, maximum difference between primers 5°C), and optimal product size of 50 base pairs (minimum 50 base pairs, maximum 100 base pairs). The default settings were used for the remaining parameters. Primers were mixed at the dilutions specified by LGC Genomics before using in KASPar assays. Plates were originally run for the optimum 38 cycles on the LGC genomics SNP line, centrifuged and then read on the Pherastar plate reader. The Pherastar files were imported into Klustercaller software for genotyping. If the plates were acceptably clustered with scoreable genotypes, an additional set of 3 cycles were added and the plates were read and imported again. This addition of 3 PCR cycles and re-reading the plates was continued until scoreable clusters were formed.

Phenotypic screening

Selfed seeds were produced from recombinant plants shown to be heterozygous by marker-assisted selection. The ratio of the recombinant in the selfed seeds will be ~ 1: 2: 1 for the tested marker. The seeds were collected, ginned, delinted and stored in an incubator at 40°C for two weeks to increase consistency of germination. Before phenotypic screening, a set of 10 selfed seeds from the selected recombinants were planted, and DNA was extracted using young, folded or newly unfolded leaves from the seedlings by the Qiagen DNeasy Plant Mini Kit. The DNA then was tested for BNL1231_null. To date, BNL1231_null is the marker closest to the *Ren^{lon}* gene, and as far as is known, it has co-segregated 100% with both reniform nematode resistance and

seedling stunting. Selfed seeds from the selected recombinant plants were used to test for stunting and reniform nematode resistance, i.e., to determine if linkage was broken between *Ren^{lon}* and nearby loci that we hypothesize could control susceptibility to stunting.

Stunting test assay

In the stunting assay, FM 966 served as a nematode-susceptible non-stunting control, and experimental line LONREN MR-19 served as a nematode-resistant stunting susceptible control. Five experimental replicates were used for each control. Ten BC1S1 progeny from each recombinant were used because they were expected to segregate 3:1 for susceptibility to stunting.

Experiments were conducted in growth chambers at the USDA-ARS, College Station, Texas. The temperature was set to 20°C during night (11 hours) and 25°C during daytime (13 hours). A fine sandy loam top soil was bought from a local dealer (Oppie's Topsoil, Sand, and Gravel; 1755 W. 28th Street, Bryan, TX 77803), and mixed 3:1 with washed sand. The wetted mixture was pasteurized with aerated steam treatment at 75°C for 16 hours on each of two consecutive days. Pasteurized soil was equilibrated in growth chambers for at least 24 hours before use.

Planting cups, drilled and fitted with fiber glass screen for drainage, were filled with about 500 g of sandy loam soil. One day before planting seedlings, a 50 g core of soil (5

cm long, 2.5 cm diameter) was replaced in the center of the cup with infested soil, which contained a mixture of fungi and reniform nematodes. The core soil mix for the stunting test contained 43.5 nematodes/gram. The nematode population was originally from Arkansas provided by R. T. Robbins, University of Arkansas.

Seeds were scarified, rinsed with hot tap water (50°C) for 20 seconds, rolled in germination paper (Andwin Scientific, Catalog Number: 28334-194) and germinated at 30°C for 24 hours, and then at 14°C for another 24 hours, to obtain radicles of consistent length (~ 5 cm). After that, seedlings were transplanted individually into cups.

Each cup was fertilized before planting by applying 50 mg of 10-30-20 fertilizer (Scotts Peters 99350 10-30-20 Water Soluble Peat Lite Plant Starter Fertilizer) in 50 ml water. After one week, 25 mg of 10-30-20 fertilizer was used. On subsequent weeks by intervals until harvest, each cup was fertilized with 50 mg of 15-5-25 fertilizer (Scotts Peters 9922015-5-25 Water Soluble Peat Lite Flowering Fertilizer) in 50 ml water. Plant height data were recorded after two weeks of planting.

Reniform nematode resistance test assay

In the reniform nematode resistance test assay, FM 966 was used as a nematode-susceptible control, and experimental line MR-19 as a nematode-resistant control. Eight experimental replicates were used for controls and BC1S2 seedlings from homozygous

BC1S1 plants selected by MAS from five BC1F1 recombinant plants. Four replicates of LONREN-2 also were included in the test.

The experiments were conducted in a growth chamber at the USDA-ARS, College Station, Texas. The temperature was set at 28°C for 13 hours of light and 22°C for 11 hours of dark. The soil mix used for nematode assays was made by mixing 90.9 liter sandy loam: 7.6 liter vermiculite, 600 ml dolomite and 300 ml gypsum. The soil was thoroughly wetted and pasteurized at 75°C for 16 hours. The soil was equilibrated in growth chambers for 24 hours before use. Seed germination and transplanting steps were the same as for the stunting test assay.

Reniform nematodes were extracted by the Baermann funnel technique from sandy loam soil infested with an Arkansas population of reniform nematode provided by R.T. Robbins. Reniform nematodes were injected into the soil surrounding seedlings using a 1 ml syringe (B-D Luer Lock Part No. 309628). Approximately 4,000 reniform nematodes were injected for each cup.

Three cores of soil from each cup were sampled eight weeks after inoculation, and extracted by the Baermann funnel technique. Then nematodes were counted and counts per gram of soil were calculated. Statistical analyses were performed using SAS (Version 9.3; SAS Institute, Cary, NC).

Results

MAS for the best recombinants

Five new recombinants each with 5 new break points between SNP markers Gl_168758 and Gl_072641 were found among 17,600 BC1F1seeds (**Table 5.3**).

Table 5.3. Five new recombinants with recombination locations by MAS.

Recombinant progeny	DCO donor	Gl_168758	BNL1231	Gl_072641	new break point
D1F3	7123	H	A	A	proximal to BNL1231
LS33C5	7112	H	A	A	proximal to BNL1231
LS53F11	7110	A	H	H	Distal to BNL1231
LS78D5	7126	A	H	H	Distal to BNL1231
LS149B11	7110	A	H	H	Distal to BNL1231

A: homozygous for *G. hirsutum* allele; H: heterozygous for *G. longicalyx* allele

BNL1231, to date, is the only marker that has completely co-segregated with resistance and susceptibility to reniform nematode and stunting. It is a dominant marker without amplification in *G. longicalyx* (BNL1231_null). Before conducting the stunting assay, ten selfed seeds were tested from each putative recombinant plant against BNL1231.

BNL1231_null was lost for two of the five recombinant lines, D1F3 and LS33C5.

Conversely, LS53F11, LS78D5 and LS149B11 still contained BNL1231_null. Stunting and reniform resistance tests were conducted next to see if any of the recombinants obtained both resistance to reniform nematode and stunting.

Stunting test assay

Plant height was measured two weeks after planting (Table 4). Progeny from D1F3 and LS33C5 did not show stunting. Progeny from LS53F11, LS78D5 and LS149B11 segregated for stunting (**Table 5.4**). The results indicate BNL1231_null was linked with stunting. A reniform resistance test was needed to determine whether BNL1231 was uncoupled from the *Ren^{lon}* gene.

Table 5.4. Plant height (cm) data two weeks after planting.

Plant	Genotypes (BC1F1 Lines or Families)						
	FM 966	MR-19	D1F3	LS33C5	LS53F11	LS78D5	LS149B11
1	8.4	3.7	9.1	6.3	6	4.1	8.1
2	6.3	3.1	6.4	8.8	8.3	7.8	3.8
3	5.8	3	8.5	8.7	6	7.1	4.2
4	7.8	3.2	7.3	7.3	4.4	5.2	9.3
5	5.5	5.4	7.8	7.6	6.5	6.1	4.2
6	n/a	n/a	6.2	9.8	6.3	5.5	5.4
7	n/a	n/a	6	9.6	3.6	7.7	9.2
8	n/a	n/a	7.7	8.7	4.9	4.1	4.2
9	n/a	n/a	7.7	9.6	5.5	5.2	4.4
10	n/a	n/a	6.3	6.7	4.1	4	3.7
Avg.	6.76	3.68	7.3	8.31	5.56	5.68	5.65
Std. dev	1.27	1	1	1.19	1.31	1.38	2.17
S.E.	0.57	0.45	0.30	0.38	0.414	0.436	0.686

Reniform nematode resistance test

Reniform nematodes per gram of soil were determined and evaluated. FM 966 and MR-19, and LONREN-2 showed significant differences for nematodes per gram of soil (**Fig. 5.2**). Both MR-19 and LONREN-2 had extremely low reniform nematodes from this experiment (~0 nematode per gram soil), which was consistent with previous results.

BC1S2 plants from LS53F11, LS78D05 and LS149B11 had extremely low reniform nematodes numbers similar to the LONREN lines. BC1S2 plants from LS33C05 had very high numbers of reniform nematode similar to FM 966 (up to ~40 nematode per gram soil). BC1S2 plants from D1F3 showed segregation for resistance, which was not expected. One plant from D1F3 showed higher numbers of reniform nematode than the rest; two plants showed very low numbers of reniform nematode similar to LONREN lines (~0 nematode per gram soil); and seven plants had relatively low reniform numbers (~1 to 2 nematode per gram soil) but not as low as those of LONREN lines.

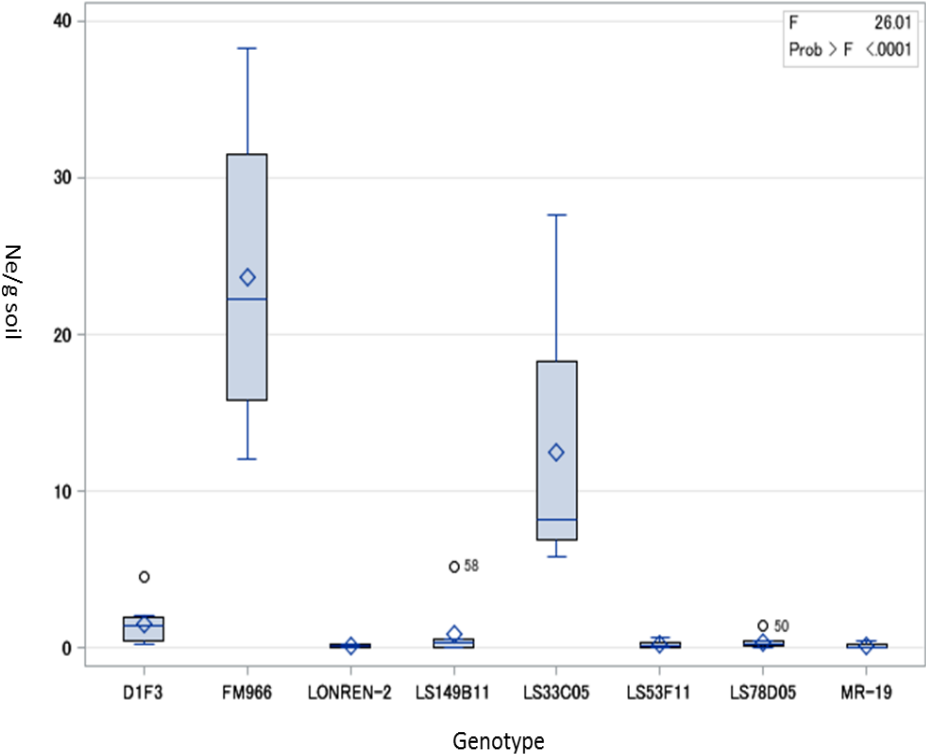


Figure 5.2. Boxplot displaying mean nematodes per gram of soil for each genotype at eight weeks after inoculation.

Discussion

In describing the reniform-resistant upland backcross products from *G. longicalyx* introgression, Robinson et al. (2007) noted that each contains an alien *Ren^{lon}*-bearing segment of unknown physical length and genetic composition, because linkage map distances (cM) are poor indicators of absolute physical size (Mbp). Given that the rate of recombination between the alien segment (F₁-genome) and c11 (A-subgenome of the [AD]₁ genome) is much lower than between normal c11 homologs, the physical size of the alien segment could be significantly under-estimated by linkage map distances (48, 124). The larger the alien c11 segments, the more likely that they would contain agronomically deleterious linked genes and perform less than optimally for one or more other significant traits. Genetic recombination might mitigate or solve the stunting issues of *Ren^{lon}* lines by differentially eliminating linked alleles that cause stunting while retaining the beneficial linked allele for nematode resistance.

Achieving desirable recombinants around *Ren^{lon}* is complicated by the low rate of recombination of the alien segment harboring *Ren^{lon}* with chromosome-11 of *G. hirsutum*, which is not completely homologous. Moreover, DNA extraction of large numbers of individuals is another challenge. A total of 17,600 testcross seeds from different resistance families and FM 966 were screened using seed DNA extraction method (Chapter III) for the two closest SNP markers. Five recombinants with reduced flanking regions were found among 17,600 seeds, which reflects the rare recombination rate between the F sub-genome and the A sub-genome in the *Ren^{lon}* region.

The existing genomic resources do not yet make it easy to discover/identify all of the SNPs linked with *Ren^{lon}*, especially the ones very close to *Ren^{lon}*, because the D5 genome is the only cotton reference sequence available at present. *Ren^{lon}* and its flanking region may be specific to *G. longicalyx*. For example, the SSR marker BNL1231_null is the closest marker to the *Ren^{lon}* gene, and no recombination events have been found between BNL1231_null and *Ren^{lon}*. The BNL1231 primers do not give amplification of a product from *G. longicalyx*, which could be due to a difference in the *Ren^{lon}*-flanking region in *G. longicalyx* and *G. hirsutum*. The two closest *Ren^{lon}*-linked SNP markers were identified according to the alignment results to the D5 genome assembly and the screening panel. The actual distance between those two SNPs and the *Ren^{lon}* gene in the F genome is unknown. The new recombinants identified by the two SNPs may not have the shortest possible alien segment.

BC1S1 progeny from each of five BC1F1 plants selected as "recombinants" were tested in the stunting assay. The results showed that for two, D1F3 and LS33C05, the BC1S1 families were free of stunting and thus of possible interest (**Table 5.5**). For the other three recombinant families, the plant height measured at two weeks after planting clearly showed segregation of stunting, and thus are less desirable recombination products.

Table 5.5. Performance of the five recombinants families in the stunting assay and resistance assay.

Recombinant progeny	Stunting assay (BC1S1)	Resistance assay (BC1S2)
D1F3	non-stunting	segregation for resistance
LS33C5	non-stunting	susceptible
LS53F11	segregation for stunting	resistance
LS78D5	segregation for stunting	resistance
LS149B11	segregation for stunting	segregation for resistance

BC1S2 plants from recombinant families that were homozygous for SNP markers were also tested for reniform nematode resistance. All of the families that showed stunting were homozygous for resistance except LS149B11, which showed one susceptible plants among the ten tested. Progeny from the non-stunting family LS33C05 were susceptible similar to FM 966. The other non-stunting family D1F3 segregated for resistance (**Table 5.5**). All of the BC1S2 plants were from the BC1S1 plants that were homozygous for the two closest SNP markers, therefore, the BC1S2 plants were expected to be homozygous for resistance. Since progeny from D1F3 segregated, there may have been incorrect classification based on the SNP markers, or recombination may have been occurred between the SNP markers and the resistance gene. That is unlikely, because of the very low recombination rate between the F and A sub genomes. Before any final conclusion can be made, further tests are needed to confirm whether stunting and resistance to reniform nematode were separated in the D1F3 family.

CHAPTER VI

EFFECTS OF THE RENIFORM NEMATODE RESISTANCE *Ren^{lon}* SEGMENT ON SEVERITY OF THIELAVIOPSIS ROOT ROT IN COTTON

Introduction

The reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) was recognized as a serious pest of cotton in 1959 (73). It has increased in importance since that time (141), and causes annual losses of approximately \$130M in the U. S. Cotton Belt, with losses of 4-5% in the states of Mississippi, Louisiana and Alabama (22, 84, 122).

Reniform nematode reduces cotton yield, boll size and lint percentage (40, 73). The life cycle of reniform nematode follows the basic pattern for sedentary plant parasitic nematodes. Vermiform females penetrate roots to establish feeding sites in the stele. The females remain sedentary semi-endoparasites during the remainder of their life cycle, with the anterior portion of the female and egg sacs on the root surface. The optimum temperature for reproduction is about 30°C, with no development at 16 or 36°C (20, 137).

Upland cotton (*Gossypium hirsutum* L.) cultivars lack significant resistance to the reniform nematodes, with only weak to moderate resistance reported (72, 125, 146, 170). In 1984, the African wild species *G. longicalyx* was reported to be the most resistant *Gossypium* species, and deemed completely resistant (170). A gene conferring "immunity" to reniform nematodes was transferred from *G. longicalyx* into upland cotton (124). Linkage of the introgressed trait with molecular and phenotypic markers

indicated that it is determined by a single dominant gene or haplotype *Ren^{lon}* in chromosome 11 (48). Two germplasm lines LONREN-1 (PI 669509) and LONREN-2 (PI 669510) from this resistance source were jointly released by the USDA-ARS, Texas A&M AgriLife Research and Cotton Incorporated (14).

When LONREN-1 and LONREN-2 were planted in nematode-infested fields, they exhibited severe stunting at two to three weeks after planting (12). It has been reported that this stunting is due to hypersensitivity to reniform nematode (4, 136). However, the stunted field-grown plants of LONREN were found to show symptoms typical of fungal root rots, and a wide array of fungal pathogens was isolated from the roots, including *Thielaviopsis basicola* (Berk. & Broome) Ferraris (syn. *Chalara elegans* Nag Raj & Kendrick) (16).

The soil borne fungus *T. basicola* is the causal agent of black root rot of cotton, an important seedling disease of upland cotton (*Gossypium hirsutum* L.). Black root rot was first described in Arizona in 1942 (78). The fungus overwinters as thick-walled chlamydospores that germinate in the presence of the host and adequate moisture (67). This fungus usually colonizes the root cortical tissue, causing dark brown to black discoloration of roots and hypocotyl (97, 99). Black root rot is most severe when soils are cool (below 24°C) and wet (above -20 joules/kg) (130, 153).

Interactions between nematodes and other plant pathogens have been documented in a number of crops (1, 2, 33, 53, 140, 142, 155, 161). Synergism between nematodes and fungal pathogens in cotton is much more common, or perhaps better documented, than associations between nematodes and other types of pathogens. In the U.S. Cotton Belt, interactions between nematodes and seedling disease pathogens are common. An increase in cotton seedling disease caused by *Rhizoctonia solani* occurs in the presence of root knot nematode, *Meloidogyne incognita* (119). Increasing populations of reniform nematode and *R. solani* results in a linear suppression in plant growth (132). *M. incognita*, reniform nematode, or *Hoplolaimus spp.* increase the severity of seedling disease caused by *Pythium*, *Alternaria*, *Glomerella*, and *Fusarium spp.* (1). *T. basicola* also interacts with *M. incognita* (71, 94, 104, 154, 155). Alone, nematodes and fungi each cause disease of a chronic nature, whereas in concert they can cause significant plant mortality. There is some evidence that infection by the root-knot nematode allows *T. basicola* access to vascular tissue that would not normally be invaded in the absence of the nematode (153). The reniform nematode also interacts with several fungal species, including *T. basicola*, to increase cotton seedling disease severity (108).

Observations by Agudelo et al. (4) and Sikkens et al. (136) led to the hypothesis that the hypersensitivity of LONREN germplasm to nematode feeding can cause stunting. However, hypersensitivity is but one of several possible explanations for the stunting (174). Another hypothesis is that stunting of LONREN materials (lines) results from

increased susceptibility to one or more soil-borne pathogens. Such susceptibility could result from root damage, and/or from increased genetic susceptibility. Black root rot is a particularly severe seedling disease and can be influenced by concomitant nematode attack, as noted above. We considered *T. basicola* to be a strong candidate as a significant factor in the stunting of LONREN genotypes bearing the introgressed *G. longicalyx* gene, *Ren^{lon}*, when grown in reniform-infested fields.

The present chapter reports the results of controlled experiments that investigate the LONREN stunting problem. Natural cotton fields harbor potential pathogens. One or more of these pathogens could contribute to the stunting phenomenon. Initial experiments indicated that genotypes with the *Ren^{lon}* gene are differentially susceptible and/or sensitive to *T. basicola* (12). Three experiments were conducted in controlled environments to determine whether the stunting is due solely to hypersensitivity to reniform nematode or due to the increased susceptibility to the soil borne pathogen *T. basicola* in the presence of reniform nematode and the *Ren^{lon}*-bearing alien segment.

Materials and Methods

Genetic materials

Five cotton genotypes were included in all three growth chamber experiments, germplasm line LONREN MR-19 (MR-19), cultivar Delta & Pineland ‘ DP 493’ (DP 493), cultivar Fibermax ‘FM 966’ (FM 966), germplasm line BARBREN-713, and breeding line M-315 RNR (PI 592514) (M 315). MR-19 is a reniform nematode-resistant

line that contains the introgressed *G. longicalyx* gene *Ren^{lon}*, and is susceptible to stunting in nematode-infested fields. It has the microsatellite marker BNL3279_114, which is closely linked to the *Ren^{lon}* gene (48). DP 493 is susceptible to reniform nematode, and was the last backcross parent used in the development of MR-19. Line BARBREN-713 is a germplasm line released in 2012 for resistance to reniform and root-knot nematodes. The reniform nematode resistance in BARBREN-713 was introgressed from *G. barbadense* accession GB-713 (11, 125). In contrast to LONREN lines with resistance from *G. longicalyx*, BARBREN-713 does not exhibit stunting in nematode-infested fields. ‘FM 966’ is susceptible to reniform nematodes, but does not exhibit severe stunting. M 315 is resistant to root-knot nematodes but susceptible to the reniform nematodes, and was the last backcross parent used in the development of BARBREN-713.

Seed germination and soil preparation

Seeds were scarified and rinsed with hot tap water (50°C) for 20 seconds, then rolled in germination paper (Andwin Scientific, Catalog Number: 28334-194) and germinated at 30°C for 24 hours, then at 14°C for another 24 hours, to obtain radicles of consistent length (~ 5 cm). After that, seedlings were transplanted individually into cups containing ~500 g soil.

A fine sandy loam top soil was bought from a local dealer (Oppie's Topsoil, Sand, and Gravel; 1755 W. 28th Street, Bryan, TX 77803), and mixed 3:1 with washed sand. The

wetted mixture was pasteurized with aerated steam treatment at 75°C for 16 hours on 2 consecutive days. Pasteurized soil was equilibrated in growth chambers for at least 24 hours before use. Natural sandy loam soil was used directly without pasteurization.

Various soil-borne pathogens including *T. basicola* were found in the natural sandy loam soil by isolating from cotton seedling roots grown in the soil for 48 hrs (12).

Experiment 1 -- *T. basicola* and reniform nematode in pasteurized sandy loam soil

The impact of reniform nematode and *T. basicola* on cotton seedlings and plant development was examined for the five genotypes described above, using pasteurized soil in growth chambers. Pathogenicity of fungal isolates was tested alone and in combination with reniform nematode. This experiment included four treatments: [i] control, untreated (CON); [ii] nematode only (NE); [iii] *T. basicola* only (TB); and [iv] reniform nematode and *T. basicola* (NE+TB) together. The experimental design was a 4 x 5 factorial randomized complete block design (RCBD). One seedling was grown per cup, each containing 500 g of soil. Nematode treatment consisted of *ca.* 2500 reniform nematodes per cup (5 reniform nematodes/g soil).

Experiment 2 -- Natural sandy loam soil

The same five genotypes were tested in the natural sandy loam soil that contained *T. basicola* in addition to other microorganisms but not reniform nematode.

Treatments were evaluated in natural sandy loam soil with nematodes present (NE) or absent (CON). The treatment design was a 2 x 5 factorial RCBD. One seedling was

grown per cup, each containing 500 g of soil. Nematode treatment consisted of *ca.* 2500 reniform nematodes per cup.

Experiment 3 -- High concentration of reniform nematodes in pasteurized sandy loam soil

The treatment design was a 2 x 5 factorial RCBD with the five genotypes and reniform nematode present (NE) or absent (CON). One seedling was grown per cup, each containing 500 g of soil. In contrast to the other two experiments, the nematode treatment consisted of *ca.* 10,000 reniform nematodes per cup (20 reniform nematodes/g soil).

All of the experiments were conducted in controlled environment chambers at the USDA-ARS Southern Plains Agricultural Research Center, College Station, Texas. In the randomized complete block experimental designs (RCBDs), each growth chamber-time combination served as a block, with 4 replications (plants per genotype) in each block. Two blocks were tested for each experiment. The temperature was set to 20°C during night (11 hours) and 25°C during daytime (13 hours).

Reniform nematode extraction

Reniform nematodes were extracted by the Baermann funnel technique from soils infested with the nematode provided by R.T. Robbins, University of Arkansas. Extracted nematodes were free of *T. basicola*, even though the fungus was occasionally found in the nematode infested soil (16).

T. basicola inocula

Isolates of *T. basicola* were obtained from roots of cotton growing in the sandy loam soil. Methods of *T. basicola* inocula preparation were similar to those of Bell et al. (12). Fungal isolates were increased on a 100g of sterile fine sand mix containing 1.5% ground cotton roots in 250 ml flasks. Flasks were infested by adding fungal spores from the periphery of 3-week old cultures growing on carrot juice medium, and then were incubated for at least a month with periodic mixing before using as inocula. Quantitative estimates of *T. basicola* inocula in the flasks were made by dilution plating on a selective medium (16). The concentration of *T. basicola* inocula obtained in this manner was about 600,000 colony-forming units/g soil.

Inoculation with T. basicola and reniform nematode

A 50 g portion of fungal culture in soil was diluted by thoroughly mixing it with 1000 g of soil to obtain the inoculum concentration added to cups. Cups were filled with about 500 g of sandy loam soil. One day before planting seedlings, a 50 g core of soil (5 cm long, 2.5 cm diameter) was replaced with the *T. basicola*-infested soil mix. For the CON treatment in experiment 1, pasteurized sandy loam soil was placed in the core area.

Seedlings were planted in the middle of the *T. basicola* infested core, one seedling per cup. Reniform nematodes were injected as a suspension using a 1 ml syringe (B-D Luer Lock Part No. 309628) with a perforated needle into the area surrounding seedlings.

Three injections were placed in a triangular pattern about 2.5 cm from the seedling, and a fourth injection was placed very close to the seedling.

Fertilization

Each cup was fertilized before planting by applying 50 mg of 10-30-20 fertilizer (Scotts Peters 99350 10-30-20 Water Soluble Peat Lite Plant Starter Fertilizer) in 50 ml water. After the first week, 25 mg of 10-30-20 fertilizer was used. Thereafter, each cup was fertilized with 50 mg of 15-5-25 fertilizer (Scotts Peters 9922015-5-25 Water Soluble Peat Lite Flowering Fertilizer) in 50 ml water every week until harvest.

Data collection and statistical analysis

Fresh shoot weight, plant height and root weight were recorded three weeks after planting. All statistical analyses were performed using SAS (Version 9.3; SAS Institute, Cary, NC). Treatment effects were analyzed within each genotype. For experiment 1, to test for different performances of each genotype among four treatments (CON, NE, NE+TB and TB), one-way ANOVA was used to analyze treatment effects for each genotype separately. If a significant F-test was detected ($P < 0.05$), the Student-Newman-Keuls' (SNK) test was used to further elucidate treatment differences ($P = 0.05$). For experiment 2 and 3, since there were only two treatments for each genotype, a t-test was used to detect treatment differences for each genotype ($P = 0.05$). Data from two repeated growth chamber experiments (trials) were pooled with four replicates in each, and trials were considered as block effects.

Results

In Experiment 1, the growth medium was pasteurized soil. Plant heights of all five genotypes were not reduced by the NE treatment, as compared to the CON treatment (**Fig. 6.1A**). In fact, BARBREN-713 plant height was increased by NE treatment, as compared to CON treatment. The TB treatment significantly reduced the plant heights of all five genotypes, as compared to the CON and NE treatments. The combined treatment, NE+TB, stunted plant height of MR-19 more than the TB treatment. MR-19 was the only genotype for which plant height was significantly reduced by the NE+TB treatment, as compared to the TB treatment. The effects of Experiment-1 treatments on shoot weights paralleled the effects on plant height, as described above. When given the NE+TB treatment, MR-19 was the only genotype for which the shoot weight decreased significantly more than that of seedlings given the TB treatment (**Fig. 6.1B**). The effects on root weight were somewhat different. Root weights of all genotypes were increased by the NE treatment, as compared to the CON treatment. The increase was significant for roots of four genotypes, but not MR-19 (**Fig. 6.1C**). For all five genotypes, the root weights were greatly reduced by the TB and NE+TB treatments, as compared to the CON and NE treatments.

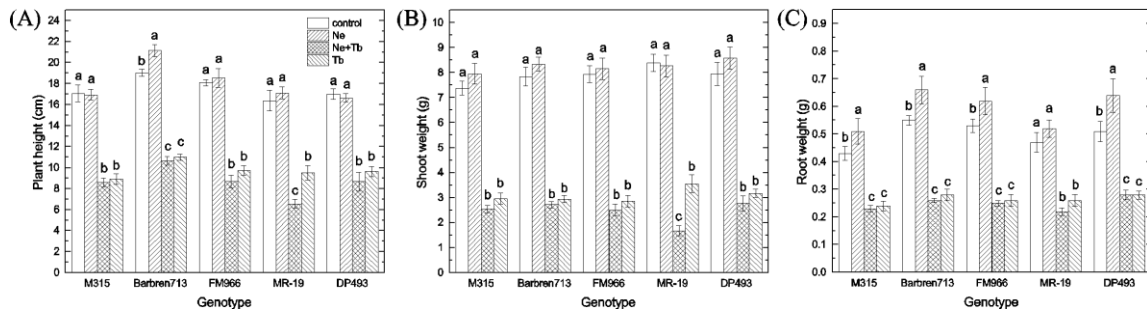


Figure 6.1. Plant height (A), shoot weight (B) and root weight (C) of plants grown in pasteurized sandy loam soil, including five genotypes (M 315, BARBREN-713, FM 966, MR-19, and DP 493) and four treatments, control (CON), reniform nematode only (NE), reniform nematode and *T. basicola* (NE+TB), and *T. basicola* only (TB). Each bar represents the mean of 8 replicates (plants) pooled from two growth chamber experiments with four replicates each and the lines indicate the standard errors of the means. Letters within each genotype indicate significant differences among treatments according to SNK test ($P < 0.05$). The concentration of reniform nematode inoculations was 5 nematodes/g soil.

The NE treatment had little effect on seedling height and shoot weight, but tended to increase root mass. The single and combined treatments with TB or NE+TB dramatically decreased seedling growth of all five genotypes. According to all three measures, the responses of all of the genotypes except MR-19 were similar to the NE+TB and TB treatments, with only slightly more severe responses to the NE+TB treatment. However, the response to the NE+TB treatment was exceptionally strong for MR-19, which displayed significantly stronger decreases for plant height and shoot weight, as well as differences in root weight, which were close to significant, too.

In Experiment 2, the growth medium was non-pasteurized natural soil. Of the five tested genotypes, only MR-19 had significantly lower shoot weight ($P = 0.0157$) after the NE treatment, as compared to the CON treatment (**Fig. 6.2A and 6.2B**). For plant height and

root weight, there were no significant differences between seedlings receiving NE or CON treatments for any of the five lines (**Fig. 6.2C**) ($P \geq 0.107$). Results of Experiment 1 were generally similar to those of Experiment 2, but less severe. When under the NE treatment, MR-19 suffered significant decreases in shoot weight, but declines in root weight and plant height were non-significant.

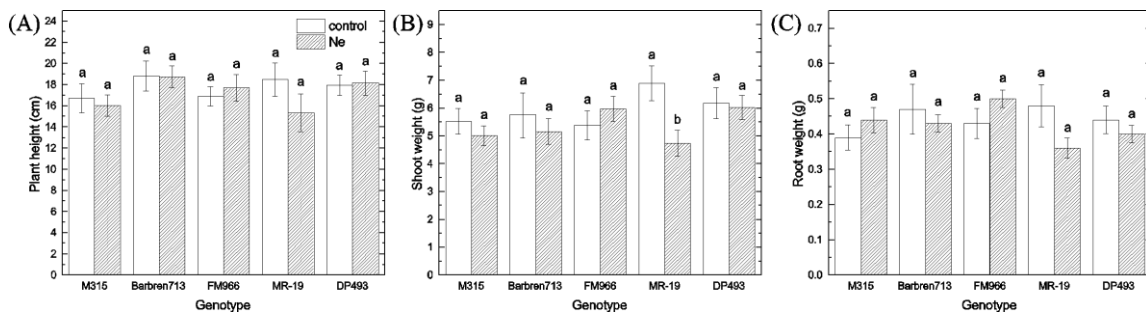


Figure 6.2. Plant height (A), shoot weight (B) and root weight (C) of natural (non-pasteurized) sandy loam soil test, including M 315, BARBREN-713, FM 966, MR-19, and DP 493 with two treatments of control (CON) or with 5 reniform nematodes/g soil (NE). Soil pathogens, including *T. basicola* were present in the natural sandy loam soil. Each bar represents the mean of 8 replicates (plants) pooled from two growth chamber experiments with four replicates each and the lines indicate the standard errors of the means. Letters within each genotype indicate significant differences among treatments and growth conditions, according to t-tests ($P < 0.05$). The concentration of reniform nematode inoculations was 5 nematodes/g soil.

In Experiment 3, the growth medium was pasteurized sandy loam soil. Plant heights and root weights of all five genotypes were not significantly affected by the NE treatment, as compared with CON ($P \geq 0.0730$ for plant height, $P \geq 0.0956$ for root weight). However, shoot weight of DP 493 was increased by the NE treatment, as compared to the CON

treatment ($P = 0.0432$). Shoot weights of all the other four genotypes were not significantly affected by NE treatment (**Fig. 6.3**) ($P \geq 0.2675$).

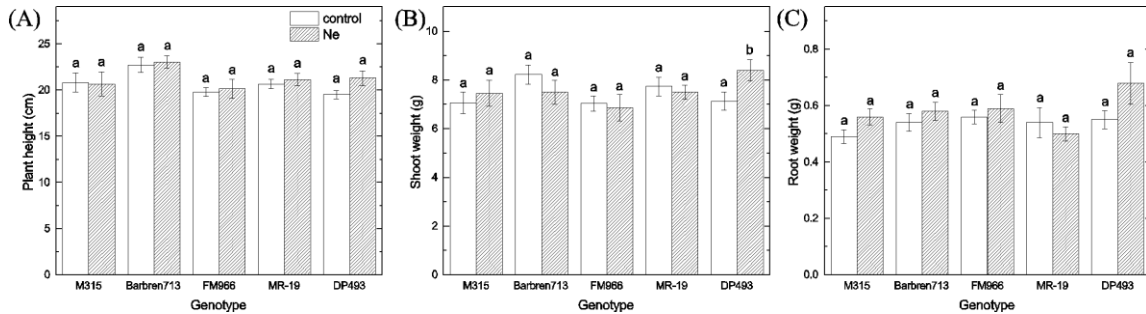


Figure 6.3. Plant height (A), shoot weight (B) and root weight (C) of plants grown in pasteurized sandy loam soil. Genotypes M 315, BARBREN-713, FM 966, MR-19, and DP 493 were grown without (CON) and with 20 reniform nematodes/g soil (NE). Each bar represents the mean of 8 replicates (plants) pooled from two growth chamber experiments with 4 replicates each and the lines indicate the standard errors of the means. Letters within each genotype indicate significant differences among treatments and growth conditions, according to t-tests ($P < 0.05$).

Discussion

The pasteurized sandy loam soil used in Experiment 1 and Experiment 3 was free of *T. basicola* and other soil-borne pathogens, greatly reducing complexities of the experiments. The natural sandy loam soil used in Experiment 2 was not pasteurized and contained an assortment of soil borne microorganisms, including *T. basicola*. This provided an opportunity to assess effects of nematodes in the presence of a complex array of other organisms and microbes, and where the natural level of *T. basicola* was much lower than in Experiment-1 treatments involving inoculation of soil with *T.*

basicola. The inoculation levels of reniform nematode (5 nematodes/g soil) used in Experiment 1 and 2 are typical of the nematode concentrations found in the nematode-infested cotton fields of the Texas A&M AgriLife Research farm near College Station, TX, where severe stunting of LONREN lines has occurred (15). The inoculum level of reniform nematode used in Experiment 3 was four-fold higher, about 20 nematodes/g soil, and provided an opportunity to assess effects of the nematode, *per se*, at a high inoculum level.

The collective response of the five genotypes to NE treatment in Experiment 1 was to grow as large, or perhaps even larger, than non-inoculated control seedlings. Thus, when no microbes were initially present in the soil, the presence of nematodes had a slightly positive or neutral effect on initial seedling growth. Similar results were also observed in Experiment 3 even with higher nematode inoculum density. In both experiments, differential responses of MR-19 seedlings to control versus low or high nematode pressures were non-significant. These data indicate that stunting of *Ren^{lon}*-containing genotypes does not result directly from nematode-induced hypersensitivity, and suggests the involvement of one or more additional factors.

Seedlings of all genotypes in Experiment 1 were stunted more by the TB treatment than by the NE treatment. All genotypes except MR-19 responded similarly to TB and NE+TB treatments, and were severely debilitated. Furthermore, all genotypes except for MR-19 were damaged only slightly more by the NE+TB treatment than by the TB

treatment. Seedlings of MR-19, however were severely damaged by NE+TB treatment. In Experiment 2, in the presence of natural soil pathogens, all genotypes except MR-19 responded similarly to control and NE treatments. In contrast, MR-19 was significantly stunted by the NE treatment. In Experiment 1 and 2, MR-19 did not exhibit unusual sensitivity to *T. basicola* or natural soil pathogens without the application of nematodes. The differential responses by MR-19 are concordant with the hypothesis that stunting arises from increased susceptibility to *T. basicola* and perhaps other soil pathogens when nematodes are present.

Plant height, shoot weight and root weight decreased for all genotypes subjected to the NE+TB treatment. This result parallels the previous report that the combination of reniform nematode and *T. basicola* induced more severe necrosis of roots and hypocotyls than *T. basicola* alone (108). In the presence of root-knot nematodes, vascular tissue colonization by *T. basicola* may exacerbate root dysfunction and cause increased seedling mortality in grower fields under specific environmental conditions (79, 153). A similar phenomenon might underlie the interactions observed for reniform nematode and *T. basicola*. The reniform nematode could render the roots of all genotypes more susceptible to *T. basicola* by physically disrupting the continuity of the endodermis that normally functions as a barrier to further fungal invasion into the root and or by down regulation of plant defense genes at the nematode feeding sites (95). Such interaction between the nematode and fungal pathogen is often indirect and occurs owing to induced modifications in the host plant (76). For MR-19, such a disruption may

be especially pronounced (4), and physically render the roots even more susceptible to infection by *T. basicola*, and perhaps other soil pathogens. Another possibility is that susceptibility to soil pathogens is heightened as a result of overlap or interactions between physiological response pathways. Plants can be predisposed to infection by soil pathogens in response to systemic metabolites produced at the site of nematode infection (76, 115). Metabolic modifications induced in MR-19 by the reniform nematode could heighten physiological sensitivity of seedlings to soil pathogens.

In these experiments, MR-19 was not stunted any more than other genotypes by reniform nematode alone and there was no apparent root necrosis. This finding differs markedly from a previous study of LONREN stunting that reported LONREN germplasm to be differentially sensitive to high-nematode populations, with hypersensitivity leading to root necrosis and plant stunting (136). It is suspected that the discordance could be due to differences in nematode population densities or purity. In this work, 5 nematodes per gram of soil in Experiment 1 and Experiment 2 was used, which is similar to local infested field levels and within the recommended population density for pot assays (122). In Experiment 3, an elevated level of 20 nematodes per gram was used, as a further test. In contrast, previously published results were based on extremely high nematode populations levels, up to 50,000/150 cc soil, i.e., ~333 nematodes/g soil (136). Nematodes can vector plant pathogens, including plant viruses (63) and plant bacteria (113), which can significantly affect the development of some disease complexes in plants. Another difference between the studies is that the duration

of the treatments. The experimental cycle in this study was only 3 weeks, which is long enough to score stunting but short enough to minimize effects of post-planting contamination. In contrast, the previous study used nearly 9 weeks (62 days). At the conclusion of the experiments presented here, roots and hypocotyls of nematode-only treatments were not discolored (Fig. 6.4), whereas roots and soil-line regions of hypocotyls were very darkly discolored in the previous study (136), strongly indicating pathogenesis by organisms other than nematodes.

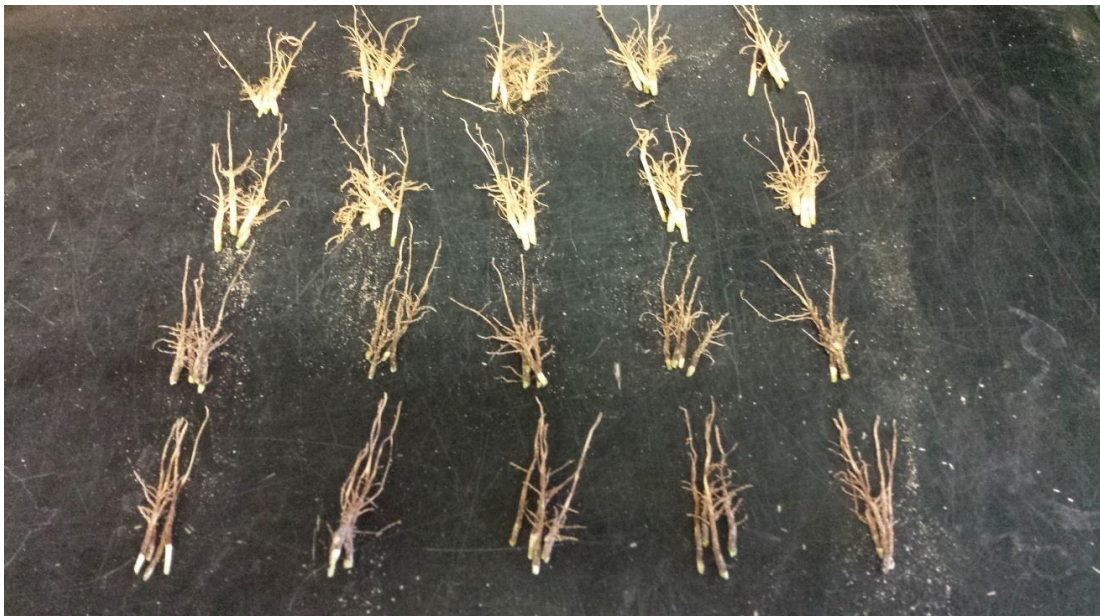


Figure 6.4. Roots of seedlings from *T. basicola* test in autoclaved sandy loam soil. From left to right are five genotypes, M 315, BARBREN-713, FM 966, MR-19 and DP 493; From top to bottom are four treatments of control (CON), reniform nematode only (NE), reniform nematode and *T. basicola* (NE+TB) and *T. basicola* only (TB).

Overall, the results indicate that stunting of LONREN genotypes containing the *Ren^{lon}* gene, e.g. MR-19, is not caused by reniform nematode alone, but is instead caused by combinations of reniform nematode with *T. basicola* and/or other natural soil-borne pathogens. Physical damage due to hypersensitivity to the nematode is at most a relatively minor factor in seedling stunting. Reniform nematodes, however, clearly exacerbate the high susceptibility of LONREN genotypes to *T. basicola* and perhaps other soil pathogens. The increased susceptibility might be due to a combination of facilitated entry past the endodermal barrier coupled with hypersensitive reactions to feeding. This would allow necrotrophic fungi to penetrate xylem tissue more readily. Additional research will be needed to sort out the basis for the increased susceptibility of cotton to black root rot, caused by the *Ren^{lon}* gene in the presence of reniform nematodes.

CHAPTER VII

CONCLUSIONS

Reniform (*Rotylenchulus reniformis*) nematodes are pathogens of cotton (*Gossypium hirsutum*) and soybean (*Glycine max.*) in the southern United States. The nematode costs US cotton growers over \$130M per year in lost yield, quality and management expenses. Extremely strong resistance was found in the wild African diploid species *G. longicalyx* and introgressed into tetraploid cotton. Two highly resistant cotton lines were released, LONREN-1 and LONREN-2, but field testing revealed severe stunting of seedlings grown in fields heavily infested with reniform nematodes. Possible explanations include root damage caused by hypersensitivity to the nematode, *per se*, and/or new or accentuated susceptibility to one or more soil-borne pathogens, e.g., due to loss of linked (repulsion) *G. hirsutum* disease resistance loci. Several nematode and fungal resistance loci of cotton are known to reside nearby. To better characterize this chromosomal region and potentially separate the resistance and stunting, a map-based approach was undertaken to recover recombinants that retain the alien resistance gene (*Ren^{lon}*) but minimize the flanking alien segments.

To better characterize the *Ren^{lon}* chromosomal region, a total of 106,795 SNPs between *G. longicalyx* and *G. hirsutum* were developed by RNA-seq. A subset of 86 *Gh_Gl* SNPs, which were randomly selected, was validated by KASPar assays, and the conversion rate was 90.70%. All the SNPs were aligned to the D5 cotton genome

assembly, and 78% of them were successfully mapped. Those SNPs will facilitate future germplasm introgression from *G. longicalyx* and analysis of products from past introgression efforts.

By aligning resistance-linked SSR markers BNL3279, BNL1231, BNL1066, and CIR316 to the D5 genome assembly, the *Ren^{lon}* region was identified in the D5 genome. All of the *Gh_Gl* SNPs that aligned to this *Ren^{lon}* region were selected and tested against a screening panel of various cotton cultivars. A total of 85 *Gh_Gl* SNPs were validated as linked with *Ren^{lon}*, of which 18 were selected for high-resolution mapping. The two SNPs most closely flanking *Ren^{lon}* were selected according to the alignment position on the D5 genome and used for MAS to identify new recombinants that would reduce the alien segments flanking *Ren^{lon}*.

To enable high-resolution mapping and large-scale screening with large populations, manual procedures for non-destructive high-throughput extraction of DNA suitable for PCR-based genotyping from cotton seeds and seedlings were developed. By sampling minimal amounts of tissue from embryonic or seedling cotyledons, damage is minimized and viability not discernibly affected, yet the yield of DNA from each seed or seedling is typically sufficient for 1000 or 500 PCR reactions, respectively. The tissue sampling procedure for seed relies on a modified 96-well plate, which is subsequently used for seed storage, and there is no need to transfer the seed, reducing chances for error. The methods allow for 1000 seed, or many thousands of seedling DNA samples to be

economically prepared per person-day and a cost near \$ 0.05 per sample. These methods are applicable before planting or pre-transplanting, as desired, and enable a wide variety of applications that require genetic testing utilizing PCR-based genotyping methods.

By using the high-throughput non-destructive seed DNA extraction method, 880 seeds from two different resistance families were mapped with 18 of the validated SNPs. A high-resolution map was partially constructed for the resistance family containing the proximal *Ren^{lon}* alien segment, but was not constructed for the resistance family containing the distal alien segment due to the very low recombination rate between *G. hirsutum* and *G. longicalyx*.

In order to identify new recombinants with minimal flanking regions, DNA was extracted from 17,600 backcross seed using the newly developed high-throughput non-destructive DNA extraction method and genotyped with the two closest flanking SNP markers. Five new recombinants with minimized flanking regions were identified from the 17,600 seeds screened. Stunting and reniform nematode resistance tests were used to evaluate selfed progeny from each recombinant to determine if any of them were resistant to reniform nematode but free of stunting. Two of the five recombinant families were free of stunting, one of which, D1F3, seemed to segregate for reniform nematode resistance, although this result requires further confirmation. If correct, it would confirm that resistance and seedling stunting have been separated through additional recombination, and that LONREN stunting is due to linkage drag.

To determine if the stunting might result directly from hypersensitivity to the nematode, and/or increased susceptibility to soil-borne pathogens, LONREN line MR-19 and four control genotypes were exposed to different treatments with the soil borne pathogen *Thielaviopsis basicola* and/or reniform nematodes. Plant height, shoot weight and root weight were used as measures of stunting. LONREN MR-19 was stunted significantly more with the combined treatment of *T. basicola* and nematodes as compared to the effect with exposure to *T. basicola* alone, and no significant stunting was observed when treated with nematodes alone. The other genotypes did not show the differential response to the combined treatment. These results indicate that the stunting of LONREN seedlings is due largely to increased susceptibility to *T. basicola* and perhaps to other soil fungi, and not due to the response to the nematode *per se*. The increased susceptibility of LONREN to stunting when facing reniform could be due secondary effects from tissue damage and/or physiological change that alter sensitivity or reaction to soil-borne pathogens.

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