MOLECULAR AND CONVENTIONAL TECHNIQUES TO DECIPHER GENETIC NETWORKS OF FIBER DEVELOPMENT TOWARD BREEDING IMPROVED COTTON GERMPLASM

by

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(Under the Direction of Andrew H. Paterson)

ABSTRACT

Study of complex biochemical pathway and genes involved in cotton fiber development is important to understanding the biology behind the longest plant cell known. Cotton seed epidermal cells go through initiation, elongation, secondary cell wall synthesis and maturation to become long lint fibers. Natural and artificial mutants are excellent tools to help decipher functions of genes involve in fiber development and/or a source to make improved germplasm. Here we used a natural mutant, *Ligon lintless-2* (*Li_2*), to identify gene/s in the fiber elongation process. In another experiment, we used mutant lines developed through EMS-mutagenesis to improve fiber quality of elite germplasm and study the effect of pyramiding novel alleles conferring improved fiber quality.

A population of 1,545 F2 plants derived from a cross between Pima S-7 (*G. barbadense*) and an *Li_2* mutant line (*G. hirsutum*) and 144 DNA markers were used to fine map the *Li_2* region on chromosome 18. We identified terminal deletion of the long arm of chromosome 18 to be the probable cause of the *Li_2* phenotype, identifying seven candidate genes. By Virus-Induced Gene Silencing (VIGS), knockdown of two genes, *GhUGT87A1-D1a* and *GhUGT87A*, showed *Li_2* like

phenotypes. This research provides new insight into the causal mutation of the *Li*$_2$ phenotype and identifies genes involved in fiber elongation.

A total of 12 mutant lines showing striking improvement in fiber attributes were studied for the stability of the mutant phenotype, heritability of the improved trait and interaction between novel alleles. A total of ten populations were developed, four involving crosses between TAM94L25 mutant lines and GA230, four from ACALA1517-99 mutant lines X GA230 and two from ACALA1517-99 mutant lines x TAM94L25 mutant lines. Each population had either a combination of two mutant lines in GA230 background or a combination of four mutant lines (ACALA1517-99 mutant lines x TAM94L25 mutant lines). Based on replicated trials in three environments we concluded that most mutant lines can be used to improve elite germplasm and thus mitigate the genetic bottleneck in cotton. In most instances, combining different mutant lines made it possible to improve two fiber attributes in a single cross.

INDEX WORDS: crop improvement, allele stacking, breeding, genomics

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DEDICATION

I would like to dedicate this work to my wife (Sejal J Patel), son (Krishav J Patel), my parents (Dr. Dahyabhai M Patel and Ranjenben D Patel), sister (Dr. Nicky N Seth), and brother-in-law (Dr. Nirav G Seth).

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

History and importance of cotton

Cotton (*Gossypium sp.*) is cultivated primarily for its fiber (BRUBAKER AND WENDEL 1994), though its edible oil makes it also a leading oilseed crop. Cotton was domesticated independently in both the Old and New World (WENDEL AND CRONN 2003; PICKERSGILL 2007; RENNY-BYFIELD *et al.* 2016). *Gossypium arboreum* and *Gossypium herbaceum* are Old World cotton species that have been cultivated for thousands of years in Africa and Asia (Viot 2017). Two New World cotton species have also been domesticated, with *Gossypium hirsutum* dated between 3400 BC to 2300 BC in Mexico, and *Gossypium barbadense* dated around 2500-1750 B.C in Peru (ABDEL-SALAM *et al.* 2009). Since the early 1900's, *G. hirsutum* has dominated world cotton production (WENDEL *et al.* 1992; CHEN *et al.* 2007).

Cotton is grown in around 100 countries with China, India and the United States of America being the top three cotton producing countries (Chapagain *et al.* 2006). Cotton is the fourth major crop in US in terms of acreage, grown in 17 states for fiber and cotton seed that generates about $8.3 billion (USDA, 2010; National Cotton Council of America. 2013). Around 80% of US cotton is exported and helps to reduce trade deficits. Most cotton fiber is used as raw material in textile industries to develop cotton clothes, towels and napkins, while some is used for home furnishing, paper, plastic, mattress padding, automobile cushions and explosives. Cotton seed are used for making cotton seed hull and cotton seed cake for livestock consumption while cotton

seed oil is consumed by human. Cotton seeds contain gossypol that is harmful to human consumption but recently cotton plants with ultra-low Gossypol Cottonseed were developed using RNAi technique, opening the door to cotton varieties with seed edible by humans (SUNILKUMAR *et al.* 2006; RATHORE *et al.* 2012; PALLE *et al.* 2013).

Genetic diversity in cotton

The cotton genus, *Gossypium*, consists of 52 species, with more being discovered. (WENDEL AND ALBERT 1992; SEELANAN *et al.* 1997; STEWART *et al.* 2015; WENDEL AND GROVER 2015; GALLAGHER *et al.* 2017). These *Gossypium* species are either diploid or tetraploid. The diploid Gossypium species have a haploid chromosome number of 13 and consist of eight monophyletic genome groups (A, B, C, D, E, F, G and K). The tetraploids are allopolyploid consisting of two diploid genomes, "A" and "D" (ENDRIZZI *et al.* 1985; PERCIVAL *et al.* 1999; WENDEL AND CRONN 2003 ; GROVER *et al.* 2007; CHEN *et al.* 2016). A total of seven allopolyploid species have been named so far, all of New World origin, including *Gossypium hirsutum* L., *G. barbadense* L., *G. tomentosum*, *G. mustelinum*, *G. darwinii*, *G. ekmanianum* and *Gossypium stephensii* (WENDEL AND GROVER 2015; GALLAGHER *et al.* 2017). Out of these seven, only *Gossypium hirsutum* L. and *G. barbadense* L. are cultivated.

Though *G. hirsutum* accounts for more than 90 % of the worldwide cotton crop, modern Upland cotton cultivars contain only half of the diversity available in *G. hirsutum* while *G. barbadense* cultivars have retained a much greater portion of available diversity, findings suggested to result from selection pressure and targeted breeding programs (WENDEL *et al.* 1992; WENDEL *et al.* 2009; TYAGI *et al.* 2014; ZHAO *et al.* 2015; AI *et al.* 2017). Research in which 250 DNA markers were screened in 320 cultivars/lines from the US National Plant Germplasm

collection found a very low level of genetic variation, lower than other major crops such as maize, sorghum, wheat and soybean (CHEE *et al.* 2004; LUBBERS *et al.* 2004).

There are natural and artificial sources available to increase the genetic diversity of Upland cotton. The natural sources can be divided into primary, secondary and tertiary gene pools based on degrees of compatibility with *G. hirsutum* (STEWART 1995; LUBBERS AND CHEE 2009). The primary gene pool consisting of seven races of *G. hirsutum*, namely, 'yucatanense', 'punctatum', 'palmeri', 'latifolium', 'marie-galante', 'morrilli', and 'richmondi' that are easily usable to increase the genetic diversity, save for some recalcitrance due to photoperiodism. (Hutchinson 1951; Lubbers and Chee 2009). Use of the secondary gene pool consisting of the remaining allopolyploid species is possible to increase genetic diversity, but progenies experience segregation distortion and carry many deleterious or unfavorable agronomical alleles. Use of the tertiary gene pool is very difficult as it contains diploid species which generally do not produce fertile hybrids in crosses with *G. hirsutum,* generally requiring chromosome doubling to be usable (LUBBERS AND CHEE 2009).

Artificial sources available to increase the genetic diversity of Upland cotton mainly consist of germplasm developed by mutation techniques or genetic modification (Genetically Modified Organisms, GMOs). Researchers have successfully release cotton mutant lines with improved fiber traits and yield components or herbicide tolerance (AULD *et al.* 2000; BECHERE *et al.* 2007a; BECHERE *et al.* 2009b; BECHERE *et al.* 2010; BECHERE *et al.* 2011; BROWN *et al.* 2012). Still mutant breeding has been rarely used in cotton breeding program due to multiple generation advancement to reduce the mutant load and stabilize the mutation.

Cotton life cycle and fiber development

Cotton is cultivated as annual crop, in contrast to the perennial nature of its wild ancestors. From planting, cotton requires around six months to be ready for harvest. During this six months some milestones of cotton development include emergence (around 3 to 5 days after planting or DAP), formation of the first true leaf (10 DAP), appearance of the first square (38 DPA), first flower (59 DPA), open bolls (120 DAP) and ready for harvesting (150 to 160 DAP) (OOSTERHUIS AND JERNSTEDT 1999). The major commercially important organ of cotton is bolls that takes about two months to open after the flower appears. These bolls contain cotton fibers that can grow up to 6 cm in some *G. barbadense* species (KIM AND TRIPLETT 2001; LEE *et al.* 2007b). Cotton fibers are unicellular unbranched cells.

The process of fiber development is complex and consist of four overlapping stages, namely, fiber cell initiation, elongation, secondary wall biosynthesis, and maturation (BASRA AND MALIK 1984). Fiber initiation starts around three to five days before anthesis and can last six to ten days (KIM AND TRIPLETT 2001; CHEN AND GUAN 2011). Many genes such as *FBP7* (auxin promoter), sucrose synthase (*Sus*), Fasciclin-Like Arabinogalactan protein, (*GhFLA1*), cotton JASMONATE ZIM-DOMAIN protein (*GhJAZ2*), and transcription factors such as *GhMYB25*, *GhMML3*, *GhMYB109*, and *GhMYB2* have been found to be involved in fiber initiation (RUAN *et al.* 2003; SUO *et al.* 2003; MACHADO *et al.* 2009; CHEN AND GUAN 2011; HUANG *et al.* 2013; GUAN *et al.* 2014; HU *et al.* 2016; WAN *et al.* 2016).

Fiber initiation is followed by fiber elongation that could last for 15 to 20 days. During this period a fiber can expand at a rate of 2mm/day (LEE *et al.* 2007b; XU *et al.* 2008). Fiber generally reaches its maximum length at the end of this process. Numerous complex and interconnected biochemical pathways participate in fiber elongation. For example, expression of

genes like *GhSCP2D* are positively related to expression of *GhPdBG3*, which controls sterol homeostasis. Maintenance of sterol homeostasis is essential for plasmodesmata permeability and fiber elongation (ZHANG *et al.* 2017). Another complex biochemical pathway is regulation of H_2O_2 and reactive oxygen species (ROS) which involve numerous genes such as *GhPK6*, Pyruvate kinase *(ZHANG AND LIU 2016)*; *GhFAnnxA*, an annexin (ZHANG *et al.* 2016a); *GhCaM7*, a calcium sensor (TANG *et al.* 2014); *GhAPX1*, ascorbate peroxidase (QIN *et al.* 2008b); *GhRac1* (KIM AND TRIPLETT 2004); *GhHD-1*, homeodomain leucine zipper (HD-ZIP) transcription factor (WALFORD *et al.* 2012) and many more. Other important processes taking place during fiber elongation regulating levels of ethylene, stress and Ca2+, water transportation, cell wall loosening, different secondary metabolic pathways, and pectin biosynthesis have been identified (HOVAV *et al.* 2008a; PANG *et al.* 2010; HAIGLER *et al.* 2012; LI *et al.* 2013; FANG *et al.* 2014; SHAN *et al.* 2014; TANG *et al.* 2014; YANG *et al.* 2014).

Cellulose (secondary wall) biosynthesis starts around 15 DPA in the middle of fiber elongation and lasts until 35 DPA. During this stage, cellulose is deposited causing cell wall thickening of up to ~3-6 μm which is highest percentage of cellulose deposition in a cell of plant kingdom (HAIGLER 2007; HAIGLER *et al.* 2009). Cellulose biosynthesis has a direct connection to fiber strength, an important component of fiber quality (PANG *et al.* 2010). Major genes expressed during cellulose biosynthesis include transcription factors (*GhMYB1* and *GhMYB7),* Endo 1,4-β-glucanase (*CEL*), Lipid transfer protein (*LTP3*) cellulose synthase (*GhCesA4*), species-specific expansin (*GbEXPATR*), and leucine-rich repeat protein kinase (*LRR RLK*) (MUNIS *et al.* 2010; KIM *et al.* 2011; MANSOOR AND PATERSON 2012; SUN *et al.* 2015; HUANG *et al.* 2016; ISLAM *et al.* 2016; LI *et al.* 2016c; FANG *et al.* 2017).

During fiber maturation, water potential of fiber decreases and minerals starts to accumulate (JOHN AND KELLER 1996). At the end of fiber development, one could expect a single fruit (boll) containing 30 to 35 seeds and around 500,000 elongated lint fibers (15 to 25% of cotton seed epidermal cells) (BASRA AND MALIK 1984; TIWARI AND WILKINS 1995; KIM AND TRIPLETT 2001; HOVAV *et al.* 2008c).

Fiber traits measure by High Volume Instrument (HVI) instrument

For many years, the major objectives of cotton breeding were biotic and abiotic stress and lint yield improvement. More recently, changes in the textile industries such as use of air jet spinning machines which are eight times faster than previous counterparts has placed greater emphasis on fiber quality (BRADOW AND DAVIDONIS 2000). There are two major instruments used to determine fiber properties, High-volume instrumentation (HVI) which generally measures bulk sample of fiber; and Advanced Fiber Information System (AFIS) which measures individual fibers (SASSER 1981; SHOFNER *et al.* 1990; SUH AND SASSER 1996; KRIFA AND ETHRIDGE 2006; KELLY *et al.* 2012). In 1960, the Plains Cotton Cooperative Association (PCCA) was a pioneer in developing HVI testing system for determining fiber properties (www.pcca.com). The major fiber quality attributes that are measured by the High Volume Instrument (HVI) are micronaire (fiber fineness), fiber length, fiber uniformity (uniformity index), fiber strength, fiber elongation, and short fiber content (SFC).

Fiber fineness or Micronaire (MIC) is determined based on the ability of compressed cotton fiber samples to allow air to pass through (www.cottoninc.com) The measurement is important because it identifies samples with coarse or immature fiber, both of which contribute to formation of ‘neps’ (small knots of entangled fibers in fabric) in yarn, thus reducing overall yarn

quality (Van der Sluijs and Hunter 1999; Ulloa 2006), and making dyeing problematic. A range of 3.7 to 4.2 of Micronaire is considered as the premium range (BRADOW AND DAVIDONIS 2000).

Fiber length or Upper Half Mean Length (UHML), measured in inches or mm, is one of the most important components of fiber quality (WAKEHAM 1955). Higher fiber length is better for high speed spinning machines as fibers can withstand more resistance. Also, higher fiber length is important for yarn quality (VAN DER SLUIJS AND HUNTER 1999; KRIFA AND ETHRIDGE 2006; ULLOA 2006). Fiber length is positively associated with multiple fiber attributes like fiber strength, uniformity and short fiber content (SFC). Thus, improvement of fiber length will affect overall fiber quality.

Fiber uniformity index is the ratio (%) of total mean length of fibers in a sample to that of the longest 50 percent of fibers in the sample (upper half mean length, or UHM). Uniformity of fiber is important for efficient yarn spinning and is positively related to yarn quality (MEREDITH 1994; ULLOA 2006).

Fiber strength is measured as the force (in grams) required to break a fiber bundle of one tex unit (COTTON 1998; HAIGLER 2010). Thus, its unit is grams/tex. Fiber strength has generally positive association with fiber length, micronaire and Fiber uniformity index. It has high impact on cotton fiber quality. It helps fiber to withstand the force generated by high speed spinning of yarn, reducing wastage and improving efficiency of fabric manufacturing. The USDA Agricultural Marketing Service (AMS), divides cotton fiber strength into various groups with 23.0 g/tex or below considered to be the lowest class and 31.0 g/tex and above the highest class.

Fiber elongation is the percentage by which a fiber could be stretched before it breaks. Fiber elongation provides the ability to withstand high throughput textile processing, adds toughness to yarn, improving yarn quality and work-to-break values (YANG AND GORDON ; WATERS *et al.*

1966). Research has suggested that there is broad scope to improve fiber elongation using conventional breeding, as it has a short breeding history (PATEL *et al.* 2014).

Short fiber content (SFC %) is measured as the percentage of fibers that are smaller than one-half inch (CUI *et al.* 2003). High SFC can cause problems in spinning, increasing irregularity in yarn, increasing wastage, number of neps and reducing strength (KRIFA AND ETHRIDGE 2006; ULLOA 2006; THIBODEAUX *et al.* 2008; CAI *et al.* 2011).

Genomic resources and QTL mapping

The basic steps to identify QTLs for a trait of interest are 1) develop a mapping population like F_2, backcross population, RILs (Recombinant Inbred Lines) or NILs (Near Isogenic Lines) 2) Phenotype the trait of interest 3) Genotype markers covering the whole genome, to permit a genome-wide scan for QTLs related to the trait.

Visible phenotypic markers, generally mutants, were used to develop the first map of the cotton genome (PERCY *et al.* 2015). Different types of markers like RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), SSR (simple sequence repeats), and SNP (Single Nucleotide Polymorphism) have been used to develop cotton linkage maps and conduct QTL mapping studies (RONG *et al.* 2004; LACAPE *et al.* 2005; RONG *et al.* 2005; RONG *et al.* 2007; YU *et al.* 2007; QIN *et al.* 2008a; XIAO *et al.* 2009; LACAPE *et al.* 2010; GORE *et al.* 2014; HULSE-KEMP *et al.* 2015; LI *et al.* 2016a; MEENA *et al.* 2017). In the 1990's RFLP markers were used to develop genetic maps (REINISCH *et al.* 1994; SHAPPLEY *et al.* 1996; SHAPPLEY *et al.* 1998). Later, a recombination map was developed using 3347 sequence-tagged site loci with average distance less than 2cM between markers (RONG *et al.* 2004). With the availability of SSRs, greater DNA polymorphism helped to construct several genetic maps (CHEN *et al.* 2008; YU *et al.* 2011). A

high density genetic map for cotton has been constructed using SSR and SNP in a RIL population derived from an interspecific cross between *G. hirsutum* and *G. barbadense* (JOHN et al. 2012). This map covers 3380 centiMorgans (cM) of the cotton genome (AD) by 2072 loci develop from 1825 SSRs and 247 SNPs.

The first QTL mapping study in cotton identified fourteen QTLs associated to different fiber traits (JIANG *et al.* 1998). QTL mapping for fiber traits in several other interspecific and intraspecific crosses has followed (ULLOA AND MEREDITH JR 2000; PATERSON *et al.* 2003; ZHANG *et al.* 2003a; MEI *et al.* 2004; RONG *et al.* 2004; LACAPE *et al.* 2005; SHEN *et al.* 2006; RONG *et al.* 2007; SHEN *et al.* 2007; QIN *et al.* 2008a; LACAPE *et al.* 2010; ZHANG *et al.* 2011; YUAN *et al.* 2014b; LI *et al.* 2016a; ZHANG *et al.* 2016b). Four meta-analyses have been performed to identify QTL hotspot and clusters for agronomically important traits (RONG *et al.* 2007; LACAPE *et al.* 2010; SAID *et al.* 2013; SAID *et al.* 2015). Recent meta-analysis used published information from 1,075 QTL and 1,059 QTL, respectively, on intraspecific crosses between *G. hirsutum* genotypes and interspecific cross between *G. hirsutum* × *G. barbadense* to identify QTL clusters and hotspots important for yield components, biotic and abiotic stress, and fiber attributes (SAID *et al.* 2015).

With availability of more genetic markers and cheaper sequencing techniques such as Genotyping by Sequencing (GBS) (DAVEY *et al.* 2011; KIM *et al.* 2016) and whole genome sequencing of reference sequences for *G. raimondii* (PATERSON et al. 2012), *G. arboreum* (LI et al. 2014a), *G. hirsutum* (LI *et al.* 2015; ZHANG *et al.* 2015), fine mapping of fiber QTLs has become easier. A QTL, *qMi-C14*, related to Root-Knot Nematode (RKN) resistance was fine mapped and 20 candidate genes were identified in the 2.3 Mb flanking region (KUMAR *et al.* 2016a). A fiber length QTL, *qFL-chr1*, was fine mapped using 1672 BC_4F_2 genotypes and 24

PCR based polymorphic markers (XU *et al.* 2017). The region was narrowed down to 0.9 cM or 2.38Mb and gene expression of two candidate genes in the region showed positive correlation with fiber length (XU *et al.* 2017). An *LRR RLK* gene was identified residing in a stable QTL, qFS07.1, associated with fiber strength (FANG *et al.* 2017). A major QTL in the vicinity of the T_1 locus on chromosome 6 was identified controlling multiple yield components, fiber quality traits and resistance to spiny bollworm (*Earias spp.*) (WAN *et al.* 2007). A large population of 6975 F_2 individuals was used to fine map the QTL to 5.3 Mb of chromosome A06 of *G. hirsutum*. By using RNA-Seq and RT-PCR, three putative genes were identified that might explain its diverse effects on yield and different fiber quality traits (LIU *et al.* 2016). The next obvious step to these fine mapping efforts will be to functionally validate the candidate genes and transfer them to elite germplasm.

Natural and Artificial Mutants

Cotton mutants containing fiber anomalies are excellent tool to decipher the complex process of fiber development and identify genes involved. Several natural and artificial cotton mutants showing striking differences from wild type have been discovered, genetically mapped and/or used to identify and map other genes related to fiber development (RONG *et al.* 2005; HINCHLIFFE *et al.* 2011; PATEL *et al.* 2014; JIANG *et al.* 2015; PERCY *et al.* 2015; HINCHLIFFE *et al.* 2016; MA *et al.* 2016; PATEL *et al.* 2016; THYSSEN *et al.* 2016; WAN *et al.* 2016; NAOUMKINA *et al.* 2017; THYSSEN *et al.* 2017).

One of the earliest discovered types of natural cotton mutants affected color, of lint, fuzz, petal spots, and leaves (SHOEMAKER 1909; MCLENDON 1912). A total of 157 morphological characteristic loci have been reported in a recent survey of cotton species (PERCY *et al.* 2015). Rong *et al.* (2005) developed six populations and mapped seven fiber mutants. They were *Li*$_1$

(KOHEL *et al.* 1992), *Li*$_2$ (NARBUTH AND KOHEL 1990), *N*$_1$ (GRIFFEE AND LIGON 1929b) and *Fbl* (KEARNEY AND HARRISON 1927) which were genetically dominant and *n*$_2$ (HARLAND 1929), *sma-4(h*$_a$*)*, and *sma-4(f*$_z$*)* (BEASLEY AND EGLI 1977) which were recessive. Today several natural mutants have been fine mapped and candidate genes have been identified, some of which have also been validated through functional analysis. The 22-bp deletion in a pentatricopeptide repeat (PPR) gene was linked to the *im* (immature fiber) mutant (THYSSEN *et al.* 2016). Natural Antisense Transcripts (NATs) produced from GhMML3_A12, a MYBMIXTA-like transcription factor 3 /GhMYB25- like gene, were identified as the cause of a naked seed mutant (N1) (WAN *et al.* 2016). Gly65Val substitution in *GhACT_LI1* (actin gene), was identified as the causal mutation of the *Li*$_1$ phenotype (THYSSEN *et al.* 2017). A 33-bp tandem duplication in the promoter region of *LATE MERISTEM IDENTITY1-D1b* (*GhLMI1-D1b*) causes higher expression of the gene and okra leaf shape in cotton (ANDRES *et al.* 2017). To identify genetic sources of brown lint fiber, 595 F_2 progenies were screened using SNPs identified between parental lines. An inversion of 1.4 Mb located just upstream of transcription factor *GhTT2_A07* causes elevated expression of the gene and was tightly linked with colored fiber (HINCHLIFFE *et al.* 2016).

Artificial or man-made mutants are generally obtained through physical, chemical or insertional mutagenesis (BALCELLS *et al.* 1991; AULD *et al.* 1992; MALUSZYNSKI *et al.* 1995; AULD *et al.* 1998; AN *et al.* 2005; CHOPRA 2005; GAO *et al.* 2006; AULD *et al.* 2009; PATHIRANA 2011). Physical mutagens like X-ray, alpha, beta, gamma, and ultraviolet radiation generally cause large and small chromosomal rearrangements (KODYM AND AFZA 2003; AULD *et al.* 2009; KRISHNAN *et al.* 2009; MBA *et al.* 2010). Examples of chemical mutagens are ethylmethane sulphonate (EMS), methylmethane sulphonate (MMS), nitrosoguanidine (NTG, NG, MNNG),

hydrogen fluoride (HF), sodium azide, N-methyl-N-nitrosourea (MNU), diethylsulfate (DES), and hydroxylamine which generally cause point mutations with rare chromosomal aberrations (AWAN *et al.* 1980; AULD *et al.* 2009; PATHIRANA 2011; DE SERRES AND HOLLAENDER 2012; TALEBI *et al.* 2012). Transposons and T-DNA are used to perform insertional mutation, with the advantage that the mutation site can be easily identified using sequence information, in a similar fashion to gene tagging by endogenous transposable elements (JEON *et al.* 2000; AHLOOWALIA AND MALUSZYNSKI 2001; JEONG *et al.* 2002; ALONSO *et al.* 2003; PATHIRANA 2011). Several phenotypic abnormalities in cotton have been discovered through different mutation techniques such as cytoplasmic sterility (NGEMATOV *et al.* 1975), photoperiod insensitivity (RAUT *et al.* 1971), trichome variation (PATEL *et al.* 2016), naked seed (BECHERE *et al.* 2009a; PATEL *et al.* 2014; KONG DEPEI 2017), imazamox herbicide tolerance (BECHERE *et al.* 2009b), glandless plants (HUSSEIN *et al.* 1982; MEHETRE AND THOMBRE 1983), short fiber (KONG DEPEI 2017; NAOUMKINA *et al.* 2017), albino cotyledon and leaves, red or violet leaves and stems, and multilayered bracts (Kong Depei 2017). Striking improvement in yield components and fiber quality was also found through physical and chemical mutagenesis (MEHETRE AND THOMBRE 1983; AULD *et al.* 2000; AULD 2000; SHAMSUZZAMAN *et al.* 2003; BECHERE *et al.* 2007a; BROWN *et al.* 2012; PATEL *et al.* 2014). Although a vast source of phenotypic anomalies generated by artificial mutation are available, scarce effort has been taken to understand genetics of the cause. Only one gene expression study has been performed to study changes in gene expression of the li_y short fiber mutant. Recently, a TILLING (Targeting Induced Local Lesions IN Genomes) database in cotton was developed by targeted sequencing of M_2 mutant lines (ASLAM *et al.* 2016). Such a database will ease the effort to identify causal mutations for striking phenotypes in mutant lines.

Review of candidate genes found in the *Li₂* region

GhIRX7_D belongs to the gene family, glycosyltransferase family 47 (ZHONG AND YE 2003; GESHI *et al.* 2010). *IRX7* or *FRA8 (FRAGILE FIBER 8)* is involved in biosynthesis of the hemicellulose glucuronoxylan that is an essential component of secondary cell walls and is particularly activated during secondary wall thickening in fibers and vessels (BROWN *et al.* 2005; ZHONG *et al.* 2005; LI *et al.* 2014b). Disrupting the gene causes reduction in secondary wall thickness, decline in amounts of cellulose and xylan, collapse of xylem vessels, decrease in stem strength, and dwarf phenotype (BROWN *et al.* 2005; ZHONG *et al.* 2005; BROWN *et al.* 2007; LEE *et al.* 2007a).

GhETO1_D encodes an ethylene-overproduction protein. In Arabidopsis, *ETO1* negatively regulates 1-aminocyclopropane-1-carboxylic acid synthase (ACS), a protein that acts as catalyst in the rate-determining step of ethylene biosynthesis (WANG *et al.* 2004; YOSHIDA *et al.* 2005; CHRISTIANS *et al.* 2009)**.** Ethylene plays an important role in fiber elongation but excessive production of ethylene might curb fiber development (SHI *et al.* 2006). Ethylene was also found to enhance cotton fiber and *Arabidopsis* root hair growth, but a tenfold increase in ethylene concentration is seen in loss-of-function *eto1* mutations, further causing shorter seedlings, smaller leaves, reduced root and petiole lengths and inflorescence sizes (CHAE *et al.* 2003; WANG *et al.* 2004; CHRISTIANS *et al.* 2009; PANG *et al.* 2010; LUO *et al.* 2014)**.** Application of abscisic acid (ABA) enormously inhibited root growth in *eto1* mutants by promoting ethylene biosynthesis (LUO *et al.* 2014). Elevated levels of ABA have been observed during fiber development of *Li₂* mutant lines, thus an increased level of ABA with reduced expression level of *GhETO1_D* during fiber elongation might have contributed to the *Li₂* phenotype (CHEN *et al.* 1997; GILBERT *et al.* 2013).

GhUBE11_Db encodes Ubiquitin-activating enzyme E1 that catalyzes the first step of three consecutive enzymatic cascades in a ubiquitination reaction which is important for biological processes such as embryogenesis, plant growth and development, hormone signaling, response to environmental stress, and senescence (HATFIELD *et al.* 1997; MOON *et al.* 2004; PICKETT 2007). Genes participating in ubiquitin-mediated protein degradation are highly up-regulated during fiber development (ZHANG *et al.* 2003b; AL-GHAZI *et al.* 2009; HO *et al.* 2010). Interestingly, regulation of ethylene biosynthesis through ubiquitin-mediated protein degradation is assisted by ethylene-overproduction protein (LYZENGA AND STONE 2012).

GhEXPA8 belongs to the expansin superfamily, wall-loosening proteins which help with cell expansion (SAMPEDRO AND COSGROVE 2005). Multiple studies have discovered the role of expansin genes during fiber development, especially during the time of fiber elongation (ORFORD AND TIMMIS 1998; JI *et al.* 2003; SHI *et al.* 2006; SHAN *et al.* 2014; BAJWA *et al.* 2015). *GhEXPA1* is one of the two genes that are directly down stream of GhHOX3, a homeodomain transcription factor involved in fiber elongation (SHAN *et al.* 2014). *GhEXPA8* was introduced into a cotton variety (NIAB 846) through Agrobacterium-mediated gene transformation. Compared to NIAB 846, stable and significant improvement in fiber length and micronaire was seen in transgenic cotton plants with higher expression of *GhEXPA8* (BAJWA *et al.* 2015).

GhUGT87A1_Da, *GhUGT87A2_D* and *GhUGT87A1_Db* are three genes belonging to Glycosyltransferase Family 1, the largest GT family (YONEKURA-SAKAKIBARA AND HANADA 2011; HUANG *et al.* 2015). UGTs (UDP-glucuronosyltransferases) participate in germination, vegetative growth, flowering, fiber development, response and signaling to biotic and abiotic stress, regulation of hormonal homeostasis, biosynthesis of secondary metabolites and their stability, and detoxification and fragmentation of endogenous compounds and xenobiotics (TAI

et al. 2008; WANG *et al.* 2012; JUAN *et al.* 2015; LI *et al.* 2016b; MAMOON REHMAN *et al.* 2016). In soybean, genome-wide analysis revealed abundant expression of UGTs during seed, shoot, inflorescence, cotyledon, meristem and other development stages (MAMOON REHMAN *et al.* 2016). *GhUGT1* might be involved in responding to osmotic stress in plants and was highly expressed in fibers and roots (TAI *et al.* 2008). *UGT87A1* and *UGT87A2* are nearly identical, and might have similar function and redundant effects. Metabolic analysis in overexpressed *UGT87A2* lines suggest the possible function of *UGT87A2* is in ascorbic acid homeostasis or cell wall biosynthesis (SAINT PAUL 2010). Compare to ugt87a2 knockout mutant line and wild type, transgenic lines with overexpression of *UGT87A2* (*87A2OE*) showed notable improvement in seed germination, survival rate and root length during abiotic stress (LI *et al.* 2016b). *UGT87A2* has ROS (reactive oxygen species) scavenging activity, thus reduced levels of superoxide, H_2O_2 and cell damage was observed in *87A2OE* compare to *ugt87a2* knock out mutant line and wild type (LI *et al.* 2016b). This indicates that one of the functions of *UGT87A2* is to regulation of ROS like H_2O_2 activities for normal growth and development of plants. Hydrogen peroxide (H_2O_2) is important for cell loosening and elongation (LISZKAY *et al.* 2004). In cotton, it has been reported to promote fiber elongation and differentiation of secondary cell walls in cotton fiber (POTIKHA *et al.* 1999; LI *et al.* 2007; QIN *et al.* 2008b; MEI *et al.* 2009). Regulation of H_2O_2 or ROS is required to avoid the arrest the fiber elongation process, as higher levels of H_2O_2 could trigger secondary cell wall biosynthesis (LI *et al.* 2007; CHAUDHARY *et al.* 2008; HOVAV *et al.* 2008b; CHAUDHARY *et al.* 2009b; GUO *et al.* 2016). Hovav *et al.* (2008) found elevated concentration of H_2O_2 in *G. herbaceum* (A genome) and *G. longicalyx* (F genome) during early fiber elongation, but the latter was not able to reduce the H_2O_2 level and thus reduced fiber elongation by enhancing stress conditions and triggering secondary cell wall biosynthesis. A

similar result was also seen in wild (short fiber) and domesticated (long fiber) cotton species (CHAUDHARY *et al.* 2008; CHAUDHARY *et al.* 2009b). Thus, it seems that *UGT87A* mediated scavenging activity of H_2O_2 during fiber elongation is important for fiber expansion.

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CHAPTER 2

IMPROVEMENT OF ELITE UPLAND COTTON GERMPLASM FOR MULTIPLE FIBER TRAITS BY TRANSFERRING NOVEL ALLELES FROM EMS-GENERATED MUTANT LINES

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Abstract

EMS-mutagenesis offers important advantages for improvement of crops such as cotton that have limited diversity in elite gene pools, as EMS-induced point mutations are less frequently associated with deleterious traits than alleles from wild or exotic germplasm. Here, we further investigated nine mutant lines out of 157 found to have significantly improved fiber properties. A total of eight populations were developed by crossing mutant lines in different combinations into GA230 (GA2004230) background. Multiple lines in each population were significantly improved for the fiber trait that distinguished the donor parent(s), demonstrating that an elite breeding line (GA230) could be improved for fiber qualities using these mutant lines. Genotypes improved for multiple fiber traits suggest that allele pyramiding may confer further improvements. Compared to mid parent values, mutant lines conferred fiber quality improvements to progeny of as high as 31.7% (O013) for micronaire, 16.1% (P058) for length, 22.4% (K92) for strength, 4.1% (Q47) for uniformity, 45.8% (N068) for elongation and 13.9% (O014) for lint percent. While further testing for phenotypic stability and estimation of yield potential is necessary, mutation breeding shows promise as an approach to reduce the genetic bottleneck of Upland cotton. The populations developed here may also contribute to identifying candidate genes and causal mutations for fiber quality improvement.

Keywords: crop improvement, functional genomics, TAM94L25, ACALA1517-99, allele stacking

Introduction

The history of cotton cultivation and improvement has included several genetic bottlenecks that constrain variation available for breeders to utilize to develop improved cultivars. A total of four Gossypium species were domesticated (WENDEL AND CRONN 2003; PICKERSGILL 2007; RENNY-BYFIELD *et al.* 2016) as sources of textile fiber, two Old World diploids (*G. arboreum L and G. herbaceum L.*) and two New World tetraploids (*G. hirsutum* and *G. barbadense*) (BRUBAKER *et al.* 1999; WENDEL AND CRONN 2003). Due to longer and stronger fiber and higher yield, tetraploid cotton species *(especially G. hirsutum)* are now preferred and account for 95% of world cotton fiber production (WENDEL et al. 1992; CHEN et al. 2007). Cotton breeding focused largely on developing high yielding and stress tolerant varieties, until increasing use of air jet and vortex spinning technologies in the textile industry motivated improving fiber quality traits (BRADOW AND DAVIDONIS 2000; MAY 2002). Major fiber quality traits measured by HVI (High Volume Instrument) or AFIS (Advanced Fiber Information System) are fiber length, uniformity, strength, fineness, maturity ratio, fiber color characteristic (color as reflectance (Rd) and yellowness (+b)) and short fiber content (KELLY et al. 2012; KELLY et al. 2015).

Intensive breeding within a narrow sampling of germplasm, together with several decades of focus on transgenic lines, has rendered the elite cotton gene pool one of the narrowest among the major crops (LUBBERS AND CHEE 2009) and has been suggested to leave very little room for fiber quality improvement (VAN ESBROECK AND BOWMAN 1998; IQBAL *et al.* 2001; PATERSON *et al.* 2004; TYAGI *et al.* 2014). Wild species can provide desirable agronomical traits and be used in breeding program to broaden the genetic diversity, but often carry deleterious alleles that must be eliminated. Moreover, reproductive barriers, meiotic drive, hardship in adaptability to new habitats and differences in photoperiodic response and flowering time, all complicate selection

within crosses involving wild relatives to achieve desired characteristics without reducing yield (ZHANG AND PERCY 2007; BARB *et al.* 2014; WAGHMARE *et al.* 2016). Cloning of alleles affecting fiber traits and transferring them into elite cotton cultivars involves prohibitive time, cost, and regulatory concerns regarding GMO (Genetically Modified Organisms).

Chemical mutagens such as EMS (Ethyl methanesulfonate) that generate single nucleotide 'point' mutations offer a means to create new alleles that are expected to be free from problems associated with linkage drag of unfavorable alleles during the cross of wild species. EMS-mutagenesis generally creates point mutation by converting G/C-to-A/T through DNA mispairing, thus reducing the chances of lethal mutation (GREENE *et al.* 2003). In a single experiment, one can use EMS to create and screen for multiple mutant lines that have significantly improved fiber qualities as compared to parental lines. In addition, one can expect to find other agronomical traits of interest such as fuzzless seeds, biotic and abiotic stress tolerance, and glabrous or hairy varieties along with improved fiber quality without affecting the lint yield (BECHERE *et al.* 2009a; PATEL *et al.* 2016). While appreciable screening of large numbers of candidate lines is necessary, one might discover genotypes with novel alleles that could contribute to desirable agronomical traits. Mutant populations can be subjected to either forward genetics or reverse genetics approaches such as TILLING to identify causal mutations (MCCALLUM *et al.* 2000). Several successes in using EMS to enhance elite cotton germplasm have been published (AULD *et al.* 2000; BROWN *et al.* 2012; PATEL *et al.* 2014).

Here we build on prior work to identify desirable EMS-mutated cotton lines (PATEL *et al.* 2014), transferring novel alleles from their source backgrounds (TAM94L25 and Acala 1517-99) to an elite breeding line, GA2004230. We also investigated the consequences of 'pyramiding' such novel alleles, in eight populations from different combinations of mutant lines crossed to

GA2004230. Significant improvement in fiber qualities of progenies developed by crosses between GA2004230 and selected mutant lines provided motivation to transfer novel alleles from mutant lines to additional elite germplasm, and more generally provided support for increased use of EMS mutants for improvement of cotton fiber quality.

Materials and Methods

Plant sources and population development

Based on initial screening of large populations of mutant lines generated using EMS in two different genetic backgrounds of *G. hirsutum*, TAM94L25 (SMITH 2003) and Acala 1517-99 (CANTRELL *et al.* 2000) in Texas (2007) and Georgia (2008), a subset of 157 mutant lines having significant improvements in one or more components of fiber quality were tested along with control lines at two locations in a replicated trial (Texas and Georgia, 2009). The data from the four environments supports that mutant lines with significantly improved fiber length, strength, elongation, fineness, lint percent and other quality and yield components can be achieved (PATEL *et al.* 2014). Nine striking mutants were selected from the 157 mutant lines showing improved fiber length (1 mutant line from Acala 1517-99), fiber elongation (1 from TAM94L24), fiber fineness (1 each from TAM94L24 and Acala1517-99), fiber uniformity (1, Acala1517-99), fiber strength (1, Acala1517-99), lint percent (1 each from TAM94L24 and Acala1517-99) and Rd value (1 from TAM94L25) (Table 1). Each of these nine mutant lines were crossed with a non-transgenic cotton variety adapted to the southeastern cotton belt, GA2004230 (PVP 201500309; from now on, GA230) (LUBBERS *et al.* 2006) to develop F1 hybrids in a greenhouse in Athens, GA (Summer 2012). The F1 hybrids were further crossed with each other to pyramid novel alleles from mutant lines contributing to various fiber traits in GA230 background (Table 2), in an off-season nursery in Mexico.

Field trial and data collection

A total of 100 BC1F1 (estimated at 50% source and 50% GA230 genetic background) progenies from each cross were grown in Watkinsville, GA in 2013. In 2014, progenies with sufficient seed were planted in a randomized complete block design (RCB) with two replicates at two locations (Watkinsville and Tifton, GA). The soil type at Watkinsville, GA was Appling Coarse Sandy Loam (fine, kaolinitic, thermic typic kanhapludults) and at Gibbs farm, Tifton, GA was Tifton loamy sand (fine, loamy, siliceous, thermic Plinthic kandiudult). A total of 35 seeds were planted in plot sizes of 3 m, spaced 1 m apart. Agronomic practices like weeding, irrigation, fertilizer application and pest management followed local recommendations to commercial growers. To obtain fiber samples, bolls were hand-picked in Athens (November 25, 2013) and Tifton (October 26, 2014) while seed cotton samples were collected from machine harvested cotton in Athens (November 19, 2014) and ginned using a 20-saw gin (DENNIS MFG. CO., INC.). Lint and seed weight (seed plus fuzz) were measured and lint percent (lint weight X 100/seed cotton weight) was calculated. Samples of 10 grams of lint were sent to Cotton Inc. to measure six fiber properties using HVI, including upper half mean fiber fineness or micronaire (MIC), fiber length (LEN), fiber strength (STR), fiber elongation (ELON), Uniformity index (UI) and Short Fiber Content (SFC).

Data analysis

Data was analyzed using **SAS 9.4** (**SAS** Institute Inc., Cary, NC, USA). The program statement “Proc CORR” was used to determine correlations between fiber traits. Heritabilities of fiber traits were calculated using parent-offspring regression by the SAS “Proc REG” statement. The contribution and significance of genotype, environment and interaction between genotype and environment for fiber traits was calculated using the SAS statement “Proc GLM”. Among

the total of eight populations, four involved crossing TAM94L25 mutant lines to GA230 (pops J, M, N, and O) and four involved crossing Acala1517-99 mutant lines to GA230 (pops K, L, P, and Q) (Table 2). The means of fiber traits of these populations were compared with the 'parental' (wild type progenitor and GA230) means using Fisher's LSD test at alpha level of 0.05 to determine if there was significant improvement in a sub-population for a fiber trait. To determine if pyramiding of two alleles for different traits produced germplasm with improvement for both fiber traits (for example MIC and ELON for pop J), Z scores for each line were calculated for each trait and the ten lines with the highest summed Z score for the two traits were compared with parental lines. The analysis considered genotype, environment, replication and selection (based on Z score of two traits) as fixed variables.

Results

Heritability between generation and correlation between fiber traits

Heritability was calculated between F2 and F3 generations for each fiber trait based on parent-offspring regression. Moderate heritability for fiber traits was seen in both populations (Table 3). In TAM94L25 X GA230 (hereafter 'TAGA') populations, LEN showed the maximum heritability of 0.43 whereas UI showed the minimum heritability of 0.17. Similarly, for Acala1517-99 X GA230 (from here ACGA) population, STR showed the maximum heritability of 0.44 whereas UI showed the minimum heritability of 0.27.

Correlation between traits helps to determine if two traits can be simultaneously improved or if improving one trait might impair another trait. Positive correlation generally suggests that two traits can be improved simultaneously, except for correlations involving MIC or SFC, for which negative values are desirable. MIC showed moderate negative correlation with fiber length and positive correlation with lint% in both populations; and small but significant negative correlation

with STR and UI in ACGA only. LEN showed moderate positive correlation with STR and UI but small negative correlation with ELON and lint%. UI had positive correlation with STR and ELONG. STR had negative correlation with ELONG in TAGA and lint% in ACGA. Moderate positive correlation between ELON and lint% was seen in both populations. SFC had negative correlation with each fiber trait except MIC and lint% (in ACGA). It had strong negative correlations (-0.79 in TAGA; -0.80 in ACGA) with UNIF and weak negative correlation of -0.17 with lint% in TAGA (Table 4).

Genotype and environment effects

ANOVA in both populations showed significant differences between genotypes and between environments but no significant genotype X environment interaction (Table 5). In TAGA the variance explained for different fiber traits by genotype ranged from 19% (ELON) to 48% (LEN), similarly in ACGA variance explained by genotype range from across trait with 21% (ELON) to 56% (LEN).

Fiber trait improvement

Improvement for MIC and ELON (Pop J)

A total of 100 lines were evaluated from 'population J', placing TAM94L25 mutants for improved MIC and ELON in GA230 background, with overall means that showed significant ($p< 0.01$) improvement over the parental lines and exceeded the midparent values by 4% (MIC) and 13.6% (ELON, Table 6). Significant improvement over the parents was realized for MIC by 30 lines with line J29 showing 19% improvement over the midparent value; and for ELON by 9 lines with line J45 showing 32.2% improvement over the midparent value (Table 6, Figure 2). The top 10 lines based on the sum of Z scores reflecting enhanced UNIF and STR showed an

average improvement of 9.9% in MIC and 25.2% in ELON relative to midparent values, each significant (Figure 3).

Improvement for LEN and STR (Pop K)

A total of 98 lines were evaluated from 'population K", placing ACALA 1517-99-derived mutants for LEN and STR in GA230 background, with overall means that showed significant improvement ($p<0.01$) over the parental lines and exceeded the mid-parent values for LEN by 5.7% and STR by 8.4% (Table 6). Significant improvement over the parents was realized for LEN by 40 lines with line K68 showing a 15.8% improvement; and STR for 54 lines with line K92 showing 22.4% improvement (Table 6, Figure 2). The top 10 lines based on the sum of Z scores reflecting enhanced LEN and STR significantly exceeded both parents and showed improvements over the midparent value of 10.7% in LEN and 16.6% in STR (Figure 3).

Improvement for MIC and lint% (Pop L & O)

A total of 89 and 87 lines were evaluated from "pop L" and "pop O", placing ACALA1517-99 and TAM94L25-derived mutants, respectively, for MIC and lint% into GA230 background. The average of individuals in Pop O showed significant improvement for MIC compared to parental lines while there was no significant difference between pop L and parents. For lint%, mean of both populations (pop L & O) were significantly better than their respective mutant sources or parent (ACALA1517-99 or TAM94L25), but not exceeding GA230. Significant improvements over parents was realized for MIC by 25 and 7 lines in pop O and pop L respectively. With respect to mid-parent MIC value, the most positive changes were in line O013 (pop O, 31.7%) and L078 (pop L, 10.4%). Likewise, for lint% significant improvement over the parents was realized by three lines in pop O with line O014 showing a 13.9% improvement and in two lines in pop L with line L071 showing a 13.1% improvement. Due to

negative association of MIC and lint% (HERRING *et al.* 2004; PATEL *et al.* 2014), only five pop O lines showed promise for improving MIC and lint%. The MIC mean of these lines was 8.2% lower (better) than the mid-parent value and lint% mean was 6.8% higher than mid-parent value (but not significantly improved over GA230). For pop L, we could identify only three lines that showed overall improvement in lint% and MIC within the population, but unfortunately their means were not significantly different from parents.

Improvement for lint% (Pop M) and Elon (Pop N)

Two populations were developed to improve lint% (pop M), Elon (pop N) and Rd value (both) in GA230 background. Mutant lines M276 (lint%), M1251 (Rd), and M2925 (Elon) used in the breeding scheme were from TAM94L25 background. Unfortunately, Cotton Inc. discontinued measurement of Rd value, so no pyramiding was further considered for these population. Based on performance of progenies in 2013 for lint% (in pop M) and Elon (in pop N), only selected genotypes from pop M (45 lines) and pop N (58 lines) were evaluated to see if there was improvement in progenies for lint% (pop M) and Elon (pop N).

The average lint% of pop M was significantly higher than TAM94L25 but not GA230. Only two lines were significantly improved over both parents, with M63 showed 11.6% improvement over the mid-parent value (Table 6). The top 10 lines for lint% in pop M showed an average 9.9% improvement over the mid-parent value and were significantly higher than both parents.

The average ELON of pop N was 19.9% higher than the mid-parent value and significantly better than both parents. A total of 11 lines showed improvement over parents, with line N068 showing the greatest improvement, 45.8% higher than the mid-parental value. The top 10 lines in the population for ELON showed an average 35.6% improvement over the mid-parent value.

Improvement for MIC and LEN (Pop P)

A total of 97 lines were evaluated from population P, placing ACALA 1517-99-derived mutants for LEN and MIC into GA230, with overall means that showed significant improvement ($p<0.01$) over the parental lines and exceeded the mid-parent values for LEN by 7.4% while MIC showed no significant improvement over parental lines (Table 6). Significant improvement over the parents was realized for LEN by 67 lines with line P058 showing a 16.1% improvement; and MIC by 18 lines with line P007 showing 12.8% improvement (Table 6, Figure 2). The top 10 lines based on the sum of Z scores reflecting enhanced LEN and MIC significantly exceeded both parents and showed improvements over the mid-parent value of 12% in LEN and 9.6% in MIC (Figure 3).

Improvement for STR and UNIF (Pop Q)

A total of 87 lines were evaluated from population Q, placing ACALA 1517-99-derived mutants for STR and UNIF in GA230 background, with overall means that showed significant improvement ($p<0.01$) over the parents and exceeded the mid-parent values for STR by 6.8% and UNIF by 1.6% (Table 6). Significant improvement over the parents was realized for STR by 36 lines with line Q85 showing a 17.9% improvement; and UNIF for 15 lines with line Q47 showing a 4.1% improvement (Table 6, Figure 2). The top 10 lines based on the sum of Z scores reflecting enhanced LEN and STR significantly exceeded both parents and showed improvements over the mid-parent value of 3.1% in UNIF and 12.6% in STR (Figure 3).

Discussion

Building on a few prior efforts that have produced improved cotton germplasm lines through EMS-generated mutations (AULD *et al.* 2000; BECHERE *et al.* 2009a; BROWN *et al.* 2012; PATEL *et al.* 2014; PATEL *et al.* 2016), from a total of 157 lines validated as possessing superior

quantitative fiber quality parameters (PATEL *et al.* 2014), here we further evaluated 9 striking mutants, confirming that 5 of the 8 (improvement of Rd value by TAM 94L25 M-1251 was not studied) mutant lines significantly improve fiber properties in a new elite genetic background (Table 6), and in most cases in two different combinations (Table 2). Further, comparison to two commercial checks (Delta Pine 393 and Fiber Max 832) showed mutants to be superior in several cases, including mean STR and LEN of pop K, STR and UNIF of pop Q, Lint_% of pop M, and LEN of Pop P (Table 6). These findings provide additional strong support for the value of these putatively novel alleles for improving fiber qualities of elite cottons.

Each of the eight test populations in the study was based on intercrossing of two F1's that involved different fiber quality mutants -- all populations contained multiple genotypes in the population improved for either one of two target traits, and from four populations (J, K, P, and Q) the average of ten lines selected based on summed z scores for the two traits were better for both traits than the parental lines (Figure 3), providing a natural foundation for pyramiding of fiber quality traits. For Pop O only five lines and for Pop L only three lines were obtained for such comparison, and the mean for MIC of pop O only was significantly improved compared to both parents and for lint% the selected lines of pop L and O were not significantly improved over the parents (Table 4). Thus, based on results from Pop O and L, it seems simultaneous improving of two traits like MIC and lint% that have negative association is arduous to achieve. Still the conclusion is based on four mutant lines, two for MIC (1 from ACALA1517-99, 1 from TAM94L25) and two for lint% (1,1) from many candidate mutant lines for MIC (18, 5) and lint% (7, 10) (PATEL *et al.* 2014) that can be further investigated. Testing more combinations with different mutant lines might identify alleles that can be used to improve both lint% and MIC in a single cross. Development of higher fiber strength and higher yield lines was possible

despite the traditional negative association between fiber strength and yield, suggesting that linkage between genes rather than pleiotropic effects cause this negative correlation (CULP *et al.* 1979; ZHANG *et al.* 2003a). On the other hand, crosses involving traits having positive correlation like LEN and STR (pop K), LEN and MIC (pop P), and STR and UNIF (pop Q) produced lines that were significantly improved for both traits. Crosses made between MIC and ELON (Pop J) showed no correlation between these traits in our population and we found genotypes that were improved for both traits (Figure 3), suggesting that simultaneous improvement of MIC and ELON is possible.

Within a population, the number of lines showing significant improvement for a trait relative to both parents ranges from two (lint% in pop L) to 64 (LEN in pop P) (Table 6). Pops L and P were developed using a common mutant line, Acala 1517-99-M3010, for MIC and Acala 1517-99-M1524 (pop L for lint%) or Acala 1517-99-M1903 (pop P for LEN). One reason for this variation may be that yield components are controlled by a more complex genetic network than fiber quality components. It can be Acala 1517-99-M1524 being relatively ineffective to improve lint% in GA230 or it might be also possible that GA230, which is elite germplasm, has less scope for improvement of lint% than fiber length. Another factor might be that some populations combine negatively associated traits like lint% and MIC, such as pop L. The two populations using mutant line Acala 1517-99-3010 also differ in the efficacy of MIC improvement, with 18 (18.6%) improved lines in pop P but only 7 (8%) in pop L.

Pops J and N were developed to improve ELON, based on mutant line TAM 94L25-2925 which previously showed 50% improvement over the parental lines control lines. Compared to mid-parental values, the top 10 lines showed average improvement of 27.6% and 35.6% for ELON in pop J and N, respectively, with the best lines showing 32.2% and 45.8%

improvement. This result continues to support the hypothesis that there is particularly large scope for improvement of ELON in elite germplasm such as GA230, perhaps because ELON has only recently become a priority in cotton breeding and has a short history of selection in elite germplasm (ZHANG *et al.* 2011).

Many lines showed remarkable improvement for additional fiber traits beyond those known to be conferred by the mutants used in population development. For example, line K68 of pop K showed improvement of 9.2% for MIC, 15.8% for LEN, 3.4% for UNIF, 19.6% for STR, and 22.7% for SFC with no effect on lint percent. Similarly, line P07 of pop P showed 12.8% for MIC, 11.75% for LEN, 3.3% for UNIF, 17.6% for STR and 19% for SFC. Such effects might be due to strong positive correlation between traits, thus mutants identified for single fiber traits might also contribute to improvement of other traits. Similar results were seen in the pilot study for mutant lines 3010 and 1903 from Acala 1517-99 background (Patel et al. 2014). Lines such as K68 and P07 that combine multiple desirable traits may warrant release as germplasm after verifying stability of the phenotypes and determining their yield potential, as done by previous cotton breeding programs in which mutant lines were crossed with cultivars to develop new germplasm (BECHERE *et al.* 2007a; BECHERE *et al.* 2011).

In summary, we validated that novel EMS-derived alleles confer improved fiber quality to elite cultivars beyond those in which they were identified, and can be combined to obtain genotypes that are improved for multiple fiber traits. Populations segregating for multiple mutations can be used to develop new germplasm and to map novel alleles conferring improved fiber quality, accelerating progress by using high-quality cotton genome sequences (PATERSON *et al.* 2012; LI *et al.* 2015) and contemporary molecular techniques like GBS (Genotype-By-Sequencing) which can provide plentiful numbers of SNPs (DAVEY *et al.*

2011). The availability of cotton genome sequence will further support the process (PATERSON *et al.* 2012; LI *et al.* 2015). Moreover, the 157 different mutant lines (103 Acala 1517-99, 54 TAM 94L-25) confirmed to be superior to parental or control lines for fiber traits including fiber length, uniformity, strength, fineness, elongation, Rd value and in some cases multiple attributes (PATEL *et al.* 2014) provide rich scope for further improvements beyond the alleles studied here, and may not represent saturation of the potential alleles that can be EMS-mutagenized.

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Tale 2.1- Mutant lines from PATEL *et al.* (2014) selected for the breeding scheme

Line #	Mean	Control mean	Parent	Selected for	Percent difference
1903	1.3	1.18	Acala 1517-99	LEN	9.60%
1793	37.1	33.8	Acala 1517-99	STR	9.70%
1524	41.4	38.7	Acala 1517-99	Lint%	7.20%
3010	4	4.71	Acala 1517-99	MIC	15.00%
2455	85.9	84.3	Acala 1517-99	UNIF	1.90%
2925	8.68	5.78	TAM 94L25	ELON	50.00%
276	43.1	40.7	TAM 94L25	Lint%	5.90%
2877	3.94	4.83	TAM 94L25	MIC	18.40%
1251	80.5	77.4	TAM 94L25	Rd value	4.10%

Table 2.2- Crossing scheme between F1 hybrids for mutant pyramiding

Pop ID	Crosses between F1 hybrid	Fiber trait targeted	Mutant parental lines	Population size
K	M1903 X GA230 M1793 x GA230	LEN X STR	Acala 1517-99	98
L	M3010 X GA230 M1524 X GA 230	MIC X Lint%	Acala 1517-99	89
P	M1930 X GA230 M3010 X GA230	LEN X MIC	Acala 1517-99	97
Q	M1793 XGA230 M2455 X GA 230	STR X UNIF	Acala 1517-99	86
J	M2925 X GA 230 M2877 X GA230	ELON X MIC	TAM 94L25	100
M	M276 X GA230 M1251 x GA230	Lint% X Rd value	TAM 94L25	45
N	M2925 X GA230 M1251 X GA230	ELONX Rd value	TAM 94L25	58
O	M2877 X GA230 M276 X GA 230	MIC X Lint%	TAM 94L25	87

Table 2.3- Heritability estimates based on parent-offspring regression for fiber quality traits in EMS-mutant-derived cotton populations

Population	MIC	LEN	UI	STR	ELON	SFC	LINT%
TAM94L25(mutants) x GA230	0.39**	0.43**	0.17*	0.30**	0.38**	0.26*	0.33**
ACALA1517-99(mutants) x GA230	0.32**	0.40**	0.27**	0.44**	0.34**	0.36**	0.41**

** and * in significant at $p < 0.01$ and at $p < 0.05$ respectively

Table 2.4-Correlations between fiber quality traits in crosses between mutant and elite cottons

	MIC	UHM	UI	STR	ELO	SFC%
TAM94L25 (mutants) x GA230						
UHM	-0.30*					
UI	0.04	0.39*				
STR	0.04	0.53*	0.44*			
ELO	0	-0.17*	0.28*	-0.07		
SFC%	-0.09	-0.42*	-0.79*	-0.51*	-0.35*	
Lint%	0.19*	-0.11*	0.19*	-0.07	0.59*	-0.17*
ACALA1517-99 (mutants) x GA230						
UHM	-0.41*					
UI	-0.10*	0.49*				
STR	-0.17*	0.58*	0.51*			
ELO	-0.01	-0.15*	0.21*	-0.12*		
SFC%	0.11*	-0.54*	-0.80'	-0.55*	-0.26*	
Lint%	0.19*	-0.19*	0.05	-0.23*	0.47*	0.01

* shows significance at $p < 0.0001$

Table 2.5- Variance components for fiber quality traits in crosses between mutant and elite cottons
(a) TAM94L25 (mutants) X GA230

	Source	DF	SS	MS	F Value	Contribution
MIC	**G**	294	115.40	0.39	2.88*	0.38
	E	2	10.89	5.45	40.01*	0.04
	G*E	586	64.58	0.11	0.81	0.21
	Error	815	110.93	0.14		
Len	**G**	294	2.86	0.01	4.27*	0.48
	E	2	0.09	0.04	18.9*	0.01
	G*E	586	1.12	0.00	0.84	0.19
	Error	815	1.86	0.00		
UI	**G**	294	858.96	2.92	1.81*	0.26
	E	2	240.79	120.40	74.77*	0.07
	G*E	586	901.25	1.54	0.96	0.27
	Error	815	1312.27	1.61		
STR	**G**	294	2256.48	7.68	2.63*	0.35
	E	2	232.37	116.19	39.83*	0.04
	G*E	586	1669.44	2.85	0.98	0.26
	Error	815	2377.29	2.92		
ELON	**G**	294	346.38	1.18	5.85*	0.19
	E	2	1219.66	609.83	3026.25*	0.66
	G*E	586	116.73	0.20	0.99	0.06
	Error	815	164.23	0.20		
SFC	**G**	294	282.57	0.96	1.68*	0.26
	E	2	90.94	45.47	79.39*	0.08
	G*E	586	250.38	0.43	0.75	0.23
	Error	815	466.79	0.57		
Lint%	**G**	294	4851.50	16.50	2.86*	0.23
	E	2	8794.74	4397.37	763.38*	0.41
	G*E	586	2994.04	5.11	0.89	0.14
	Error	815	4694.74	5.76		

* shows significance at $p < 0.0001$, G= genotype and E=environment

(b) ACALA1517-99 (mutants) X GA230

	Source	DF	SS	MS	F Value	Contribution
MIC	**G**	373	108.81	0.29	2.53*	0.35
	E	2	10.35	5.18	44.82*	0.03
	G*E	744	78.75	0.11	0.92	0.25
	Error	973	112.39	0.12		
Len	**G**	373	4.44	0.01	5.8*	0.56
	E	2	0.05	0.02	11.8*	0.01
	G*E	744	1.49	0.00	0.97	0.19
	Error	973	2.00	0.00		
UI	**G**	373	1781.84	4.78	3.24*	0.38
	E	2	425.27	212.63	144.04*	0.09
	G*E	744	1082.83	1.46	0.99	0.23
	Error	973	1436.38	1.48		
STR	**G**	373	5356.63	14.36	5.14*	0.51
	E	2	435.49	217.75	78*	0.04
	G*E	744	2053.16	2.76	0.99	0.19
	Error	973	2716.28	2.79		
ELON	**G**	373	416.47	1.12	5.72*	0.21
	E	2	1206.64	603.32	3089.75*	0.61
	G*E	744	168.78	0.23	1.16	0.09
	Error	973	189.99	0.20		
SFC	**G**	373	464.47	1.25	2.92*	0.38
	E	2	111.07	55.53	130.2*	0.09
	G*E	744	246.34	0.33	0.78	0.20
	Error	973	415.03	0.43		
Lint%	**G**	373	6888.53	18.47	3.53*	0.28
	E	2	8890.88	4445.44	849.4*	0.36
	G*E	746	3730.77	5.00	0.96	0.15
	Error	973	5092.29	5.23		

* shows significance at $p < 0.0001$

Table 2.6 - Comparison between parentals or checks and populations from crosses between elite cotton line GA230 and mutants for fiber quality traits

Pop id	Trait	Pop mean	Bkgrd (Acala or TAM)	GA230	Midparent (Bkgrd + GA230 avg.)	% improvement over midparent	Fiber Max 832	Delta Pine 393	Total # of improved lines in pop	Best line	% improv to mid-parent
ACALA1517-99 (mutants) X GA230											
K	LEN	1.21	1.12*	1.17*	1.15	5.7	1.14*	1.13*	40	k68	15.8
K	STR	32.31	29.15*	30.48*	29.82	8.4	30.6*	30.3*	54	k92	22.4
L	MIC	4.57	4.54	4.55	4.55	0.6	4.26¥	4.57	7	L078	-10.4
L	Lint%	40.56	38.01*	41.04	39.53	2.6	40.2	39.5	2	L071	13.1
P	LEN	1.23	1.12*	1.17*	1.15	7.4	1.14*	1.13*	67	P058	16.1
P	MIC	4.46	4.54	4.55	4.55	-1.9	4.26¥	4.57	18	P007	-12.8
Q	STR	31.83	29.15*	30.48*	29.82	6.8	30.6*	30.3*	36	Q85	17.9
Q	UNIF	84.18	82.11*	83.6*	82.86	1.6	83*	82.7*	15	Q47	4.1
TAM94L25 (mutants) XGA230											
J	ELON	5.36	4.48*	4.96*	4.72	13.6	5.11	5.28	9	J45	32.2
J	MIC	4.36	4.53*	4.55*	4.54	-4	4.26	4.57*	30	J29	-19
M	Lint%	42.2	38.93*	41.04	39.99	5.5	40.2*	39.5*	2	M63	11.6
N	ELON	5.66	4.48*	4.96*	4.72	19.9	5.11*	5.28	11	N068	45.8
O	MIC	4.32	4.53*	4.55*	4.54	-4.8	4.26	4.57*	25	O013	-31.7
O	Lint%	41.13	38.93*	41.04	39.99	2.9	40.2	39.5*	3	O014	13.9

Parental or checks cells with "*" are significantly inferior to population average by $p<0.01$ and ¥ are significantly superior to population average by $p<0.01$

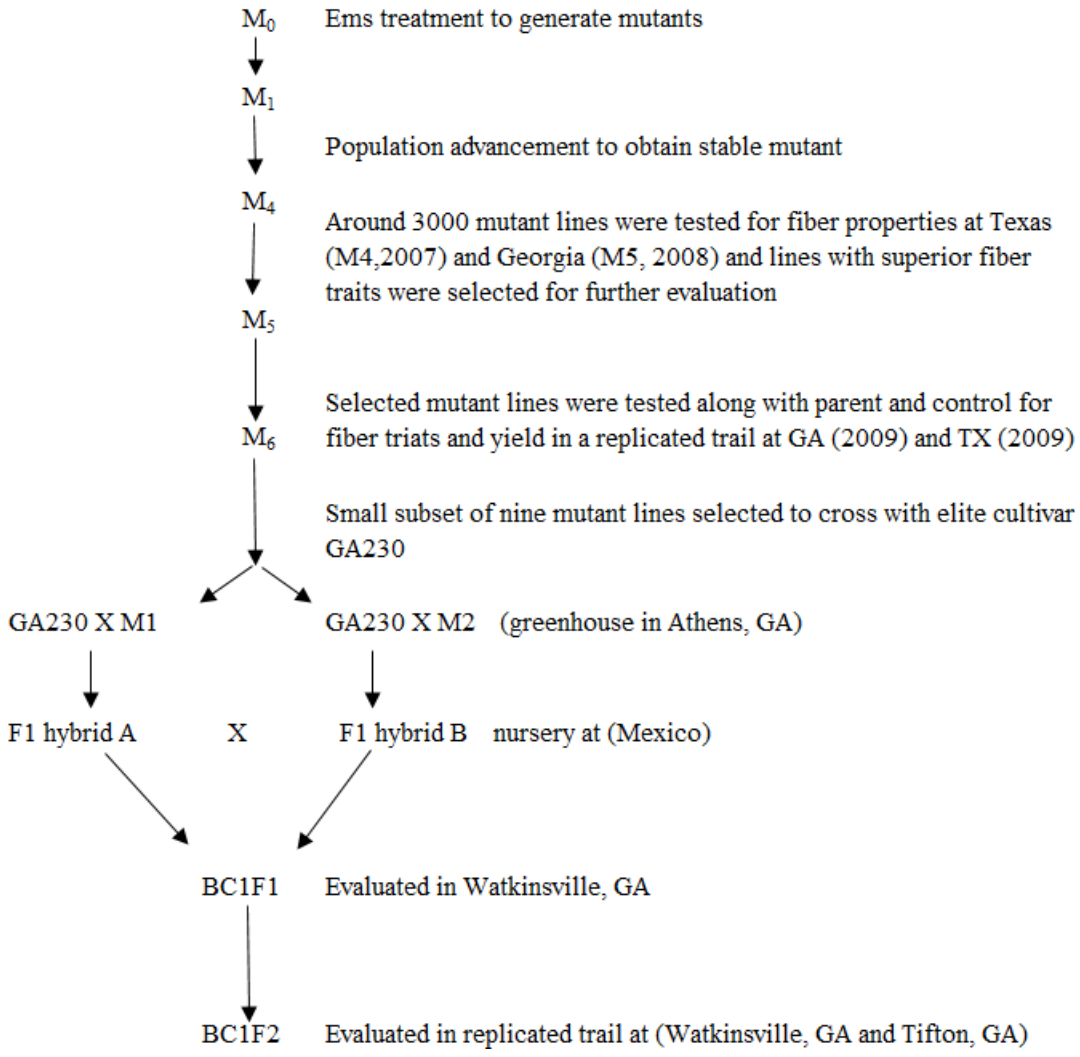


Figure 2.1- Development of populations for evaluation of multiple EMS-induced mutants

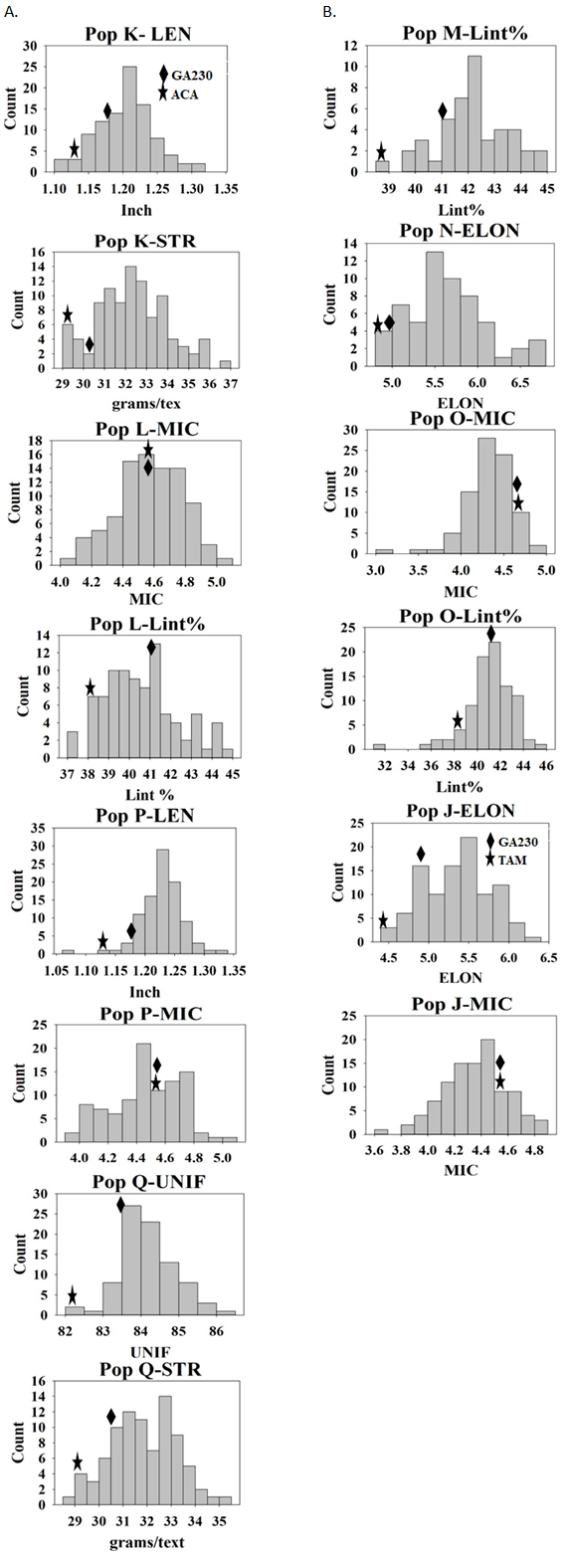
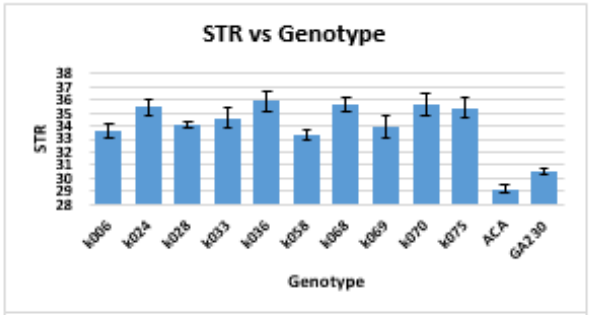
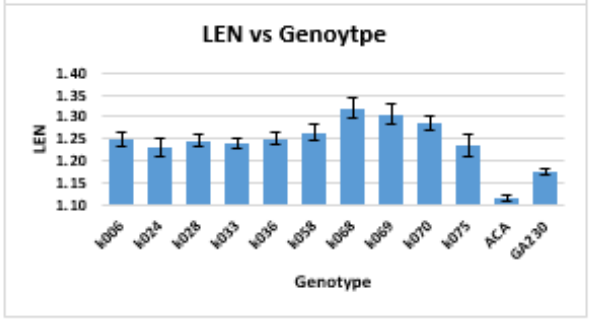


Figure 2.2 - Distribution of genotypes in populations for fiber traits they were developed. (A) populations from ACALA1517-99 (mutant) X GA230 and (B) populations from TAM94L25 (mutant) X GA230

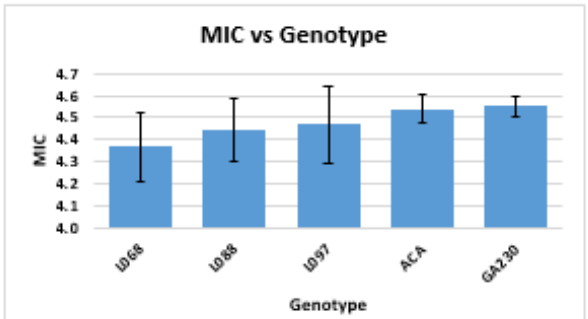
A.

a.


STR vs Genotype
STR
38
37
36
35
34
33
32
31
30
29
28
k006
k024
k028
k033
k036
k058
k068
k069
k070
k075
ACA
GA230
Genotype


LEN vs Genoytpe
LEN
1.40
1.35
1.30
1.25
1.20
1.15
1.10
k006
k024
k028
k033
k036
k058
k068
k069
k070
k075
ACA
GA230
Genotype

b.


MIC vs Genotype
MIC
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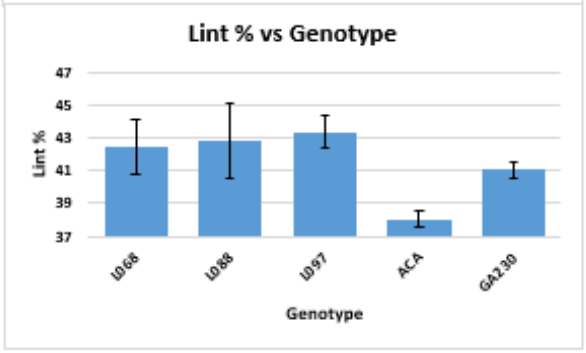
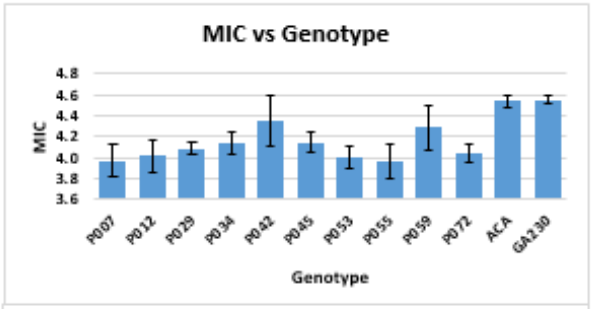
4.6
4.5
4.4
4.3
4.2
4.1
4.0
L068
L088
L097
ACA
GA230
Genotype


Lint % vs Genotype
Lint %
47
45
43
41
39
37
L068
L088
L097
ACA
GA230
Genotype

c.


MIC vs Genotype
MIC
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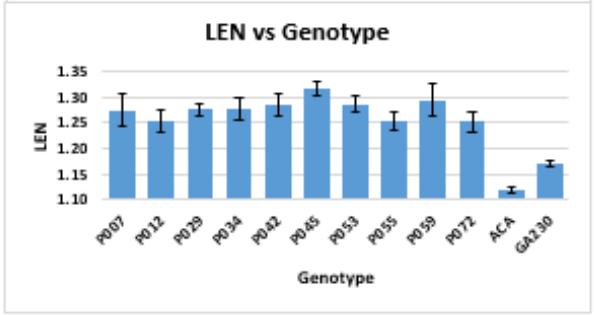
4.6
4.4
4.2
4.0
3.8
3.6
P007
P012
P029
P034
P042
P045
P053
P055
P059
P072
ACA
GA230
Genotype


LEN vs Genotype
LEN
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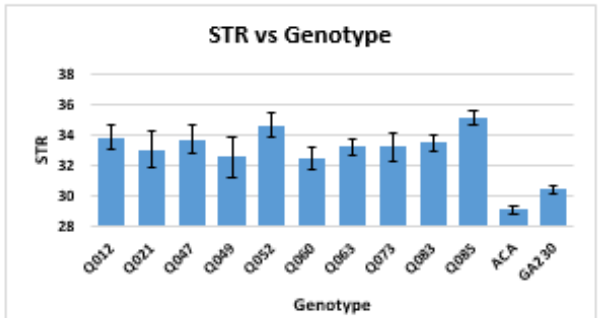
1.30
1.25
1.20
1.15
1.10
P007
P012
P029
P034
P042
P045
P053
P055
P059
P072
ACA
GA230
Genotype

d.


STR vs Genotype
STR
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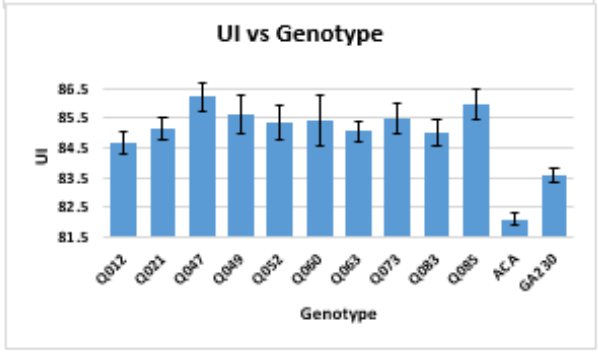
36
34
32
30
28
Q012
Q021
Q047
Q049
Q052
Q060
Q063
Q073
Q083
Q085
ACA
GA230
Genotype


UI vs Genotype
UI
86.5
85.5
84.5
83.5
82.5
81.5
Q012
Q021
Q047
Q049
Q052
Q060
Q063
Q073
Q083
Q085
ACA
GA230
Genotype

B.

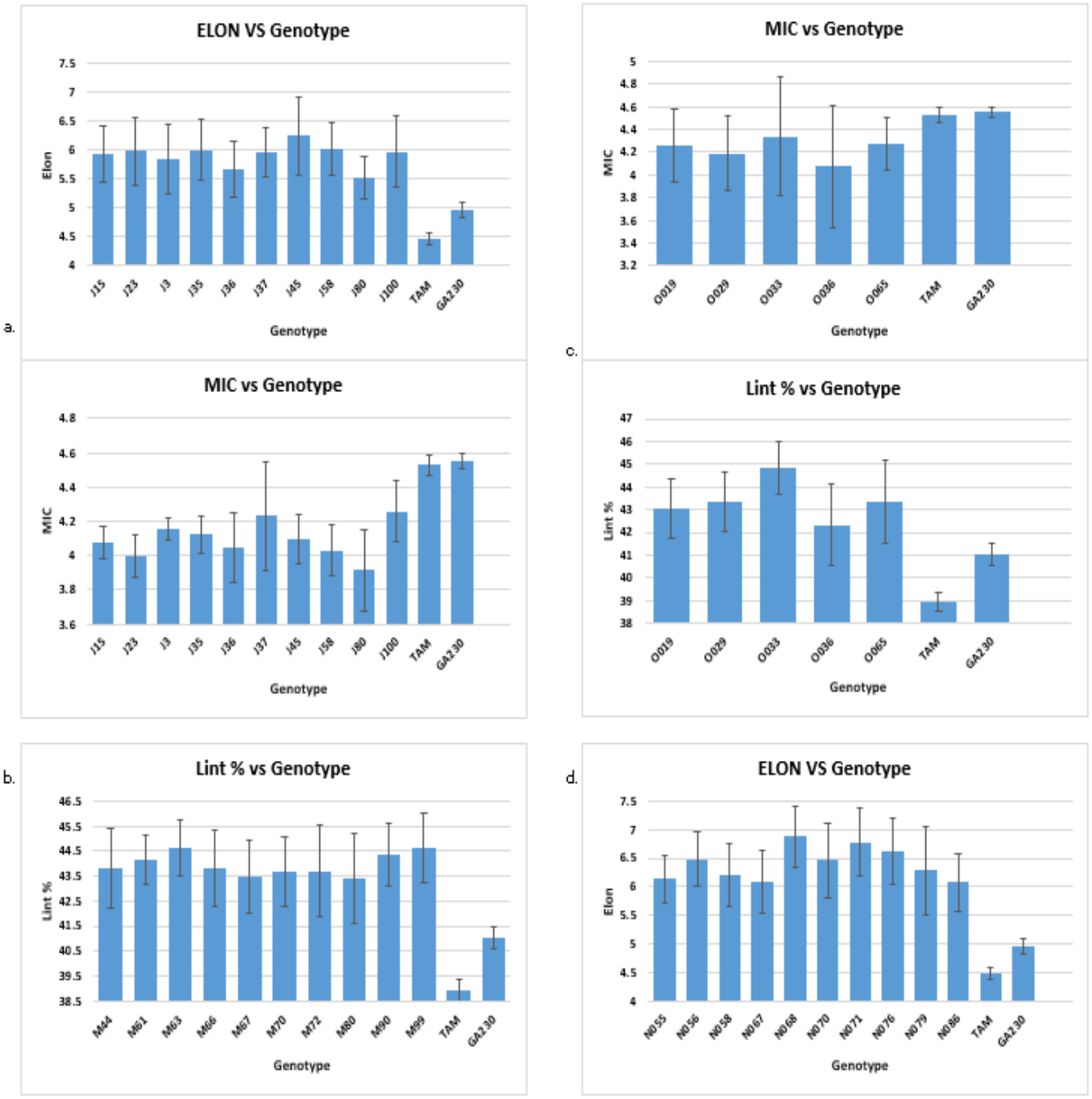


Figure 2.3 - Selected lines showing improvements over parental lines for multiple fiber traits (A) ACALA1517-99 (mutant) X GA230; and (B) TAM94L25 (mutant) X GA230. In graphs A-a is pop K, A-b is pop L, A-c is pop P, A-d is pop Q and B-a is pop J, B-b is pop M, B-c is pop O, and B-d is pop N. Error bar indicates standard error (S.E)

Chapter 3

PYRAMIDING NOVEL EMS-GENERATED MUTANT ALLELES TO IMPROVE FIBER QUALITY COMPONENTS OF ELITE UPLAND COTTON GERMPLASM.

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Abstract

Improvement of cotton fiber quality, hampered by historical genetic bottlenecks, may benefit from the use of EMS-induced mutants that are largely free of linked unfavorable alleles often associated with the use of secondary and tertiary *Gossypium* gene pools. Here we intercrossed seven of 157 EMS-generated improved fiber quality mutant lines to produce two populations, one (pop R) focused on improving four fiber attributes (micronaire, length, strength and elongation) and the other (pop S) to pyramid novel alleles for fiber length alone. The overall average of both populations was significantly improved for micronaire, fiber length, fiber strength, uniformity and short fiber content compared to parental lines, with 39 lines in pop. R and 71 in pop. S showing significant improvement for four or more traits. Lines R048, S003, S042 and S050 showed improvement for all six fiber traits tested. Fiber length of populations S and R was significantly higher than EMS mutated parent (ACALA1517-99, TAM94L25), local elite germplasm (GA230) and other commercial checks (DeltaPine 393 and Fiber Max 832). As expected average fiber length of pop. S was significantly higher by 4.2% than pop. R. Surprisingly, pop S was also significantly better than pop R in micronaire, fiber strength, uniformity and short fiber content, adding further support to hypotheses about the complex nature of cotton fiber QTLs and the corollary that selection for one fiber quality trait may also increase values of other traits. New alleles from these mutant lines show promise for improving fiber qualities beyond the levels of current elite varieties.

Keywords: crop improvement, functional genomics, EMS mutagenesis, allele stacking, genetic diversity

Introduction

Despite its global importance in contributing about one-third of the raw material used by textile industries and its cultivation in about 100 countries (CONSTABLE *et al.* 2015), allotetraploid Upland cotton (*Gossypium hirsutum L.*) improvement has been constrained by several historical genetic bottlenecks (ULLOA AND MEREDITH JR 2000; PATERSON *et al.* 2004; TYAGI *et al.* 2014; BOOPATHI AND HOFFMANN 2016). These bottlenecks are results of polyploid formation, domestication, human movement of small germplasm samples and intensive breeding for yield components (LUBBERS AND CHEE 2009).

Further increasing the need for new genetic variation, the era of high throughput rotor and air jet spinning machines in the textile industry (BRADOW AND DAVIDONIS 2000) motivated increased focus on cotton fiber quality parameters including length, strength, uniformity, micronaire and elongation, in addition to long-standing efforts to improve yield and yield components. Fiber fineness (micronaire) and elongation have particularly short histories of selection, making it rare to find an elite line with a high concentration of favorable alleles suitable to improve such attributes (ZHANG *et al.* 2011; PATEL *et al.* 2014).

Chemical mutagens such as EMS (Ethyl methanesulfonate) that generate single nucleotide 'point' mutations offer a means to create new alleles that are expected to experience fewer problems associated with linkage drag of unfavorable alleles than alleles introgressed from wild species (REINISCH *et al.* 1994; TANKSLEY AND MCCOUCH 1997; BARB *et al.* 2014; WAGHMARE *et al.* 2016). Researchers have identified EMS mutations conferring discrete morphological traits such as trichome variations (PATEL *et al.* 2016), naked seed (BECHERE *et al.* 2009a; PATEL *et al.* 2014; KONG DEPEI 2017), short fiber mutants (KONG DEPEI 2017; NAOUMKINA *et al.* 2017), albino cotyledons and leaves, red-violet leaves and stems, and multilayered bracts (KONG DEPEI

2017). Multiple years of field trials showed that mutant lines with superior fiber properties can be developed through mutation breeding (AULD *et al.* 2000; BECHERE *et al.* 2007b; BROWN *et al.* 2012; PATEL *et al.* 2014) but only a handful of attempts have been made to transfer EMS alleles into elite backgrounds and none to our knowledge have investigated combining such alleles in elite or mutant backgrounds (BECHERE *et al.* 2007b; BECHERE *et al.* 2011).

Experiments on gene or QTL pyramiding have been conducted in different plant species with a major focus on developing lines resistant to biotic or abiotic stresses (GREGORIO *et al.* 2002; ATKINSON AND URWIN 2012). In cotton, QTL pyramiding have been reported to improve fiber qualities such as fiber length and strength (WANGZHEN *et al.* 2005; DONG *et al.* 2009; YUAN *et al.* 2014b). In our prior work, we discovered multiple mutant lines that were improved for fiber length, strength, elongation, fineness, uniformity, Rd value and lint percent (PATEL *et al.* 2014). Here, we developed two populations, one combining four mutant lines that have shown improvement for multiple fiber traits (fiber length, strength, fineness and elongation) and another combining four mutant lines that all had strikingly improved fiber length. Such populations have allowed us to investigate interactions between different fiber traits, effects of allele pyramiding for the same or different fiber traits, and the possibility to break negative associations between yield and fiber quality components by crossing novel alleles generated by EMS mutagenesis.

Materials and Methods

Plant sources and population development

A total of seven mutant lines (Table 1) were used to develop two populations. Four lines, namely Acala 1517-99-M1903 (fiber length, herein abbreviated LEN), Acala 1517-99-M1793 (fiber strength, STR), TAM94L25-M2925 (fiber elongation, ELON), and TAM94L25-M2877(micronaire, MIC) were used to develop ‘pop R’; and four lines, namely, Acala 1517-99 -

M1903, Acala 1517-99 -3028, TAM94L25-M926, and TAM94L25-M2888 showing improved LEN were used to develop pop S. These lines were selected from a set of 157 mutant lines in two different genetic backgrounds [*G. hirsutum* viz. TAM94L25 (SMITH 2003) and Acala 1517-99 (CANTRELL *et al.* 2000)] that showed striking improvement over wild type progenitor ('parent') or control lines. The pilot results were supported by replicated trials in multiple environments (PATEL *et al.* 2014). In a greenhouse at Athens, GA (Summer, 2012), two mutant lines were crossed with one another to develop F1's and in an off-season nursery in Tecoman, Mexico (Winter, 2012), the F1 hybrids were further crossed with each other, thus developing populations that behave like F2 and combine four mutant lines (Table 2).

Field trial and data collection

A total of 100 F2 progenies from each population were grown in Watkinsville, GA (soil type-fine, kaolinitic, thermic typic kanhapludults) in May 2013. In 2014, a total of 95 individuals from pop R and 94 from pop S were evaluated at two locations with two replications (i.e., Watkinsville and Tifton), in a randomized complete block design (RCB). The soil type at Watkinsville, GA was Appling Coarse Sandy Loam (fine, kaolinitic, thermic typic kanhapludults) and at Gibbs farm, Tifton, GA was Tifton loamy sand (fine, loamy, siliceous, thermic Plinthic kandiudult). For all three environments both parents TAM94L25 and ACALA1517-99, TXA (TAM94L25 x ACALA1517-99), plus three checks GA230 (PVP 201500309), Fiber Max 832 (PVP 9800258), and Delta Pine 393 (PVP 200400266) were replicated 10 times for each replication in the field. A total of 35 seeds were planted in a single-row plot of 3m, with plots spaced 1 m apart. Agronomic practices like weeding, irrigation, fertilizer application and pest management were conducted as per standard cotton growing practices. To obtain fiber samples, bolls were hand-picked in Athens (November 25, 2013) and

Tifton (October 26, 2014) while seed cotton samples were collected from machine harvested cotton in Athens (November 19, 2014) and ginned using a 20-saw gin (DENNIS MFG. CO., INC.). Lint weight and seed weight (seed plus fuzz) were measured and lint percent (lint weight X 100/seed cotton weight) was calculated. Samples of 10 grams of cotton fiber were sent to Cotton Inc. to measure HVI fiber properties, namely upper half mean fiber fineness or micronaire (MIC), fiber length (LEN), fiber strength (STR), fiber elongation (ELON), Uniformity index (UI) and Short Fiber Content (SFC).

Data analysis

Data was analyzed using SAS 9.4 (**SAS** Institute Inc., Cary, NC, USA). The program statement, "Proc CORR" was used to determine correlations between fiber traits. Heritabilities of fiber traits were calculated using parent-offspring regression by the SAS "Proc REG" statement. The contribution and significance of genotype, environment and interaction between genotype and environment for fiber traits was calculated using the SAS statement "Proc GLM". The means of each fiber trait of pop R and pop S were compared with means of wild type progenitors, checks and each other by Fisher's LSD test at alpha level of 0.01 to identify significant differences. Only, feasible number of lines from a population which eliminates error of by chance improvement are carried forward for further testing and releasing as germplasm. The average of the top 10 lines for the focal fiber trait(s) of the respective populations was compared with TXA (TAM94L25 X ACALA1517-99) to see percentage gains in these lines and assess their potential merit in breeding programs to improve fiber traits. For the analysis, genotype, environment, replication and selection (top 10 lines) were considered fixed variables.

Results

Heritability of fiber traits and association between the fiber traits

Parent-offspring regression was done to calculate heritability between F2 and F3 for each fiber trait. Overall Lint_% showed the lowest heritability (0.25) while LEN showed highest heritability (0.44) (Table 3). Trends of heritability for different fiber traits were consistent with previous reports (HERRING *et al.* 2004).

Correlation coefficients were used to study association between fiber traits. In both populations, positive correlation was seen between LEN and UNIF, LEN and STR, and STR and UNIF, indicating that simultaneous improvement of LEN, STR and UNIF is possible. For MIC and SFC, a negative correlation of these traits with other fiber traits is favorable as low values of each of these traits are preferred. In both populations, SFC showed negative correlation with LEN, UNIF, STR and ELON, which means improving one or more of these fiber traits may also improve SFC. There was a negative correlation between MIC and LEN in both populations. In pop R, no correlation was observed between MIC and STR, MIC and SFC, and MIC and lint%. In pop S, there was favorable correlation between MIC and STR, and MIC and SFC; and unfavorable correlation between MIC and lint%. In pop R, ELON and UNIF had no correlation but a positive correlation in pop S. lint% was positively correlated with ELON in both populations. STR had minor negative correlation to lint% in pop S (Table 4).

Analysis of Variance

Analysis of variance in both populations showed significant difference between genotypes and between environments but no significant genotype X environment interaction. In pop R, the contribution of genotype to overall variance was lowest for ELON (19.3%) and highest for LEN

(50.5%) and in pop S, contribution of genotype to overall variance was lowest for ELON (21.5%) and highest for MIC (48.8%) (Table 5).

Fiber traits

MIC (fiber fineness)

Both populations showed significant improvement for MIC when compared to TAM (TAM94L25), ACA (ACALA1517-99), TXA and DP (Delta Pine393), but no significant difference was found between these populations and FM (Fiber Max 832). The overall mean of Pop R showed 4.5 % improvement compared to TXA, and pop S showed 7.3% improvement with significantly better MIC than pop R. Totals of 23 and 49 lines of pop R and pop S respectively showed significant improvement over both parents and TXA. The greatest improvements were shown by R093 in pop R (17.2%) and S079 in pop S (19.4%: Table 6).

LEN, UNIF, STR AND SFC

Both populations showed significant improvement for LEN, UNIF, STR and SFC with respect to all parents and checks, with the average of pop S significantly better than pop R for LEN, UNIF, STR and SFC. Compared to TXA, pop R showed an average 3.4%, 1.3%, 6.4%, and 8.8% improvement for LEN, UNIF, STR and SFC, respectively; while pop S showed 7.8%, 2.1%, 12.8% and 13.7%. The number of lines exceeding parental values for LEN, UNIF, STR and SFC, respectively was 55, 43, 38, and 59 for pop R; and 78, 74, 87, and 87 for pop S. The best lines for LEN, UNIF, STR and SFC, respectively, were R001 (10.5% improvement), R073 (4%), R090 (16.6%), and R073 (19.5%) in pop R; and S044 (14.9% improvement), S027 (4.3%), S018 (21.8%), and S044 (24.8%) in pop S (Table 6).

ELON

For ELON, pop R showed significant improvement compared to TAM and TXA, but no significant difference from ACA and the two elite checks. Pop S showed significant improvement over TAM but no significant improvement over TXA and FM, and was significantly inferior to ACA, DP and pop R. Compared to TXA, the mean of pop R showed 15.3% improvement whereas pop S showed 5.2% improvement. No line in either population had significantly higher ELON than ACA, but 39 and 9 genotypes in pop R and pop S, respectively, showed significant improvement over TXA. Line R038 of pop R showed 32% improvement compared to TXA while S025 showed 21.3% improvement.

Lint %

Both populations had significantly lower lint% compared to all the parental and checks. Average of pop R was 6.5% lower and pop S was 7.7% lower than TXA. No line in either population was significantly better than the parental lines. However, 60 and 41 genotypes in pop R and pop S, respectively, had improved fiber quality and were not significantly different for lint% than the parental lines.

Discussion

Building on recent evidence that EMS-induced mutants may contribute substantially to mitigating a lack of genetic diversity owing to genetic bottlenecks during cotton evolution, domestication, selection and crop breeding practices (BECHERE *et al.* 2007b; BROWN *et al.* 2012; PATEL *et al.* 2014), the present research validates additional EMS-mutants for roles in cotton fiber quality (beyond what were validated in a companion study, Patel et al. unpublished) and explores the effects of pyramiding multiple mutants.

Surprisingly, pop S, successfully combining multiple mutations to improve LEN alone, also had better MIC, STR, UNIF and SFC than pop R, combining mutants to improve LEN, MIC, STR and ELON, which suggests that it is possible to improve multiple fiber attributes by targeting single fiber quality traits such as LEN. This might be due to the presence of fiber QTL hotspots comprised of dozens of genes with coordinated expression during different stages of fiber development (PATERSON *et al.* 2012). Thus, by editing a single gene through EMS-mutagenesis or other mechanisms, we might affect the function of other genes that might produce pleiotropic effects. There might be a negative effect of one of the mutant lines TAM94L25-M2925 (selected for ELON) which was used to develop pop R as ELON generally have negative association with other fiber traits.

SFC and UNIF were not directly targeted in this breeding program (although SFC is clearly related to LEN) but we still found striking improvement in both mutant populations compared to all parents and checks. SFC is a major factor contributing to irregularity in yarn and reducing its strength (THIBODEAUX *et al.* 2008; CAI *et al.* 2011). The number of neps (small knot of entangled fibers in fabric) that reduce the overall quality of yarn is also positively associated with SFC (VAN DER SLUIJS AND HUNTER 1999; ULLOA 2006). Here we found lines such as R073 and S44 with reduced SFC content by 19.5% and 24.8% respectively with respect to TXA, that can be used to improve yarn quality.

Lint % is an important component of cotton yield. Pop R had better lint% than pop S, but both populations had lower lint% than parents and checks. This was expected as yield components are strongly negatively associated with fiber quality (MEREDITH 1984; CLEMENT *et al.* 2012; CONSTABLE *et al.* 2015). Still, multiple lines in both populations had improved fiber qualities with no adverse effect on lint%, suggesting that negative association between fiber traits

and yield components could be overcome, as also suggested in previous research (CLEMENT *et al.* 2015). Intermating among such lines coupled with recurrent selection may weaken negative associations and produce lines with superior fiber qualities and adequate Lint%. Similar strategies have been suggested by CLEMENT *et al.* (2012) to break negative correlations between yield components and fiber quality.

Multiples lines in each population showed improvement for more than one fiber trait. In pop S, 79% of total lines in the population showed significant improvement in at least four fiber traits over TXA, with 29 lines were improved for four fiber traits, 39 for five fiber traits, and three (S3, S42 and S50) for six fiber traits (LEN, STR, UNIF, MIC, ELON and SFC), with multiple lines showing no significant difference for ELON and lint% when compared to parental and TXA. This further supports our hypothesis that breeding programs using such mutant lines for one fiber trait can simultaneously improve other fiber traits. For pop R, 41% of total lines in the population showed significant improvement over TXA in at least four fiber traits, with 22 lines improved for four fiber traits 16 for five and one (R048) for six (LEN, STR, UNIF, MIC, ELON and SFC).

A few lines stood out as being particularly well suited for direct use to improve fiber quality in mainstream breeding programs. For example, genotype S050 of pop S showed striking improvement of 14.1% in MIC, 13.5% in LEN, 3.8% in UNIF, 20.1% in STR, 16.1% in ELON and 23% in SFC content compared to TXA but had significantly lower lint%. Genotype S032 showed improvement of 12.6% for MIC, 12.7% in LEN, 2.8% in UNIF, 17.4% in STR, 11.8% in ELON and 21.8% in SFC and no difference in lint% with respect to TXA. Similarly, S046 showed improvement of 15.9% for MIC, 12.1% in LEN, 2.3% in UNIF, 13.2% in STR and 18% in SFC and no difference in ELON and lint% with respect to TXA. In pop R, R021 showed improvement of 6.8% in LEN, 2.1% in UNIF, 12.5% in STR, 21.3% in ELON, and 14.7% in

SFC content compared to TXA and line R090 showed 12.9% in MIC 6.2% in LEN, 2% in UNIF, 16.6% in STR, and 12.9% in SFC content with respect to TXA. Such lines were not significantly different for lint% as compare to TXA. Thus, lines like R021, R090, S032, S046 and other genotypes showing improvement in multiple fiber traits with no adverse effect on lint% can be further tested for yield trials and for stability of the improvement before releasing them as germplasm lines.

In summary, the present research shows the opportunity for simultaneously improving multiple traits and the merit of pyramiding independent EMS-induced mutants for a trait. The overall mean of pop S was better for LEN, MIC and STR than pop R and more lines with improvement in multiple fiber traits were found in pop S than R, despite that pop R was developed to improve four fiber traits (MIC, LEN, STR and ELON) simultaneously while pop S was developed to improve LEN alone. Further work is needed to determine if this is a general trend or peculiar to these particular sets of mutants, and to investigate consequences for yield components and other traits. It would of course be interesting to identify such mutants that presumably have pleiotropic effects on multiple fiber traits. The ability to manipulate germplasm containing discrete mutations affecting fiber traits provides new insight into cotton breeding strategies, that may inform fiber improvement programs using natural or induced alleles, as well as CRISPR/CAS9 genome editing (DOUDNA AND CHARPENTIER 2014; SHALEM *et al.* 2015).

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Table 3.1- Superior fiber quality mutant lines selected from PATEL *et al.* (2014) for population development

Mutant id	Mean	Control mean	Significance	Background	Improvement in	% improv.
926	1.26	1.15	0.0001	TAM 94L25	LEN	9.00%
1903	1.3	1.18	0.0001	Acala 1517-99	LEN	9.60%
2888	1.25	1.15	0.0001	TAM 94L25	LEN	8.60%
3028	1.27	1.18	0.001	Acala 1517-99	LEN	7.20%
2925	8.68	5.78	0.0001	TAM 94L25	ELONG	50.00%
2877	3.94	4.83	0.0001	TAM 94L25	MIC	18.40%
1793	37.11	33.84	0.005	Acala 1517-99	STR	9.70%

Table 3.2- Crossing scheme of F1 hybrids to study effect of combination of different novel alleles on fiber traits

Pop id	Crosses between F1 hybrid	Fiber trait targeted	Mutant parental lines	Population size
Pop R	1903-1 X 2925-1 2877-2 X 1793-1	(LEN +ELON) X (MIC + STR)	Acala 1517-99 + TAM 94L25	95
Pop S	926-4 X 3028-2 2888-1 X 1903-3	LEN	Acala 1517-99 + TAM 94L25	94

Table 3.3- Parent-offspring regression estimates of heritability for seven cotton fiber traits across two mutant-containing populations

	Athens 14	**Tifton 14**	**Total_14**
MIC_A13	0.21	0.37	0.29
LEN_A13	0.46	0.42	0.44
UNIF_A13	0.28	0.3	0.29
STR_A13	0.36	0.39	0.37
ELON_A13	0.34	0.31	0.33
SFC_A13	0.37	0.46	0.41
Lint %_A13	0.21	0.3	0.25

Table 3.4-Correlations between seven cotton fiber traits in two mutant-containing populations

	MIC	**UHM**	**UI**	**STR**	**ELO**	**SFC %**
Pop R						
UHM	-0.24*					
UI	0	0.50*				
STR	0	0.55*	0.54*			
ELO	-0.10	-0.15	0.16	-0.08		
SFC	-0.03	-0.53*	-0.83*	-0.56*	-0.24*	
Lint %	0.14	-0.08	0.12	0.11	0.39*	-0.11
Pop S						
UHM	-0.50*					
UI	-0.10	0.47*				
STR	-0.21*	0.40*	0.44*			
ELO	0.12	-0.13	0.35*	0.10		
SFC	0.26*	-0.70*	-0.80*	-0.47*	-0.28*	
Lint %	0.39*	-0.22*	0.06	-0.19*	0.45*	0.03

* shows significance at $p < 0.0001$

Table 3.5- Variance components for seven cotton fiber traits across two mutant-containing populations

Fiber traits	Source	DF	SS	MS	F Value	% Contribution	DF	SS	MS	F Value	% Contribution
		Pop R					Pop S				
MIC	G	94	31.3	0.33	3.20*	33.6	93	44.4	0.48	4.27*	48.8
	E	2	17.6	8.8	84.40*	18.9	2	4.95	2.47	22.12*	5.4
	G*E	185	24.6	0.13	1.28	26.4	186	20.69	0.11	0.99	22.7
	Error	190	19.8	0.1			188	21.03	0.11		
Len	G	94	0.65	0.01	4.23*	50.5	93	0.78	0.01	4.01*	45.3
	E	2	0.04	0.02	11.89*	3.0	2	0.08	0.04	19.64*	4.8
	G*E	185	0.29	0	0.95	22.4	186	0.46	0	1.19	27.0
	Error	190	0.31	0			188	0.39	0		
UI	G	94	269	2.86	2.11*	32.8	93	290.26	3.12	2.04*	29.4
	E	2	51	25.5	18.79*	6.2	2	155.76	77.88	50.84*	15.8
	G*E	185	243	1.31	0.97	29.6	186	252.16	1.36	0.88	25.6
	Error	190	258	1.36			188	288	1.53		
STR	G	94	832	8.85	2.85*	41.6	93	635.8	6.84	2*	32.5
	E	2	52.9	26.5	8.52*	2.7	2	75.97	37.99	11.1*	3.9
	G*E	185	523	2.83	0.91	26.2	186	598.83	3.22	0.94	30.6
	Error	190	590	3.11			188	643.59	3.42		
ELON	G	94	77.8	0.83	4.89*	19.3	93	55.04	0.59	3.37*	21.5
	E	2	257	129	760*	64.0	2	136.31	68.15	389*	53.3
	G*E	185	34.9	0.19	1.11	8.7	186	31.4	0.17	0.96	12.3
	Error	190	32.2	0.17			188	32.98	0.18		
SFC	G	94	61.6	0.65	2.05*	31.1	93	82.49	0.89	2.5*	33.4
	E	2	16.2	8.09	25.35*	8.2	2	36.74	18.37	51.7*	14.9
	G*E	185	59.5	0.32	1.01	30.1	186	61.02	0.33	0.92	24.7
	Error	190	60.7	0.32			188	66.8	0.36		
Lint %	G	94	1012	10.8	1.35*	20.9	93	1370.1	14.73	2.62*	26.5
	E	2	1264	632	79.51*	26.1	2	1574.6	787.28	140*	30.5
	G*E	185	1058	5.63	0.71	21.9	186	1163.8	6.26	1.11	22.5
	Error	190	1502	7.95			188	1057.7	5.63		

Table 3.6 – Comparing of each fiber trait between two population, parental and check lines

Pop id	Trait	Pop mean	TAM	ACA	TXA	% improvement compare to TXA	GA 230	Fiber Max 832	Delta Pine 393	Total # of improved lines in	Best line	% improv to TXA	Difference in % to pop R
R	MIC	4.3	4.53*	4.54*	4.5*	4.5	4.55*	4.26	4.57*	23	R093	17.2	..
R	LEN	1.19	1.14*	1.12*	1.15*	3.4	1.17*	1.14*	1.13*	55	R001	10.5	..
R	UNIF	83.72	82.33*	82.11*	82.66*	1.3	83.6*	82.96*	82.69*	43	R073	4	..
R	STR	31.83	30.44*	29.15*	29.9*	6.4	30.48*	30.62*	30.33*	38	R090	16.6	..
R	ELON	5.36	4.48*	5.37	4.65*	15.3	4.96	5.11	5.28	0	R038	32	..
R	SFC %	7.7	8.57*	8.67*	8.44*	8.8	7.88*	8.35*	8.17*	59	R073	19.5	..
R	Lint %	36.59	38.93¥	38.01¥	39.11¥	-6.5	41.04¥	40.17¥	39.49¥	0	R010	5.5	..
S	MIC	4.17	4.53*	4.54*	4.5*	7.3	4.55*	4.26	4.57*	49	S79	19.4	-3*
S	LEN	1.24	1.13*	1.12*	1.15*	7.8	1.17*	1.14*	1.13*	78	S44	14.9	4.2*
S	UNIF	84.39	82.33*	82.11*	82.66*	2.1	83.6*	82.96*	82.69*	74	S27	4.3	0.8*
S	STR	33.72	30.44*	29.15*	29.9*	12.8	30.48*	30.62*	30.33*	87	S18	21.8	5.9*
S	ELON	4.89	4.48*	5.37¥	4.65	5.2	4.96	5.11	5.28¥	0	S25	21.3	-8.8¥
S	SFC %	7.28	8.57*	8.67*	8.44*	13.7	7.88*	8.35*	8.17*	87	S44	24.8	-5.5*
S	Lint %	36.09	38.93¥	38.01¥	39.11¥	-7.7	41.04¥	40.17¥	39.49¥	0	S24	2.2	-1.4¥

Parental or checks cells with "*" are significantly inferior to population average by $p<0.01$ and "¥" are significantly superior to population average by $p<0.01$

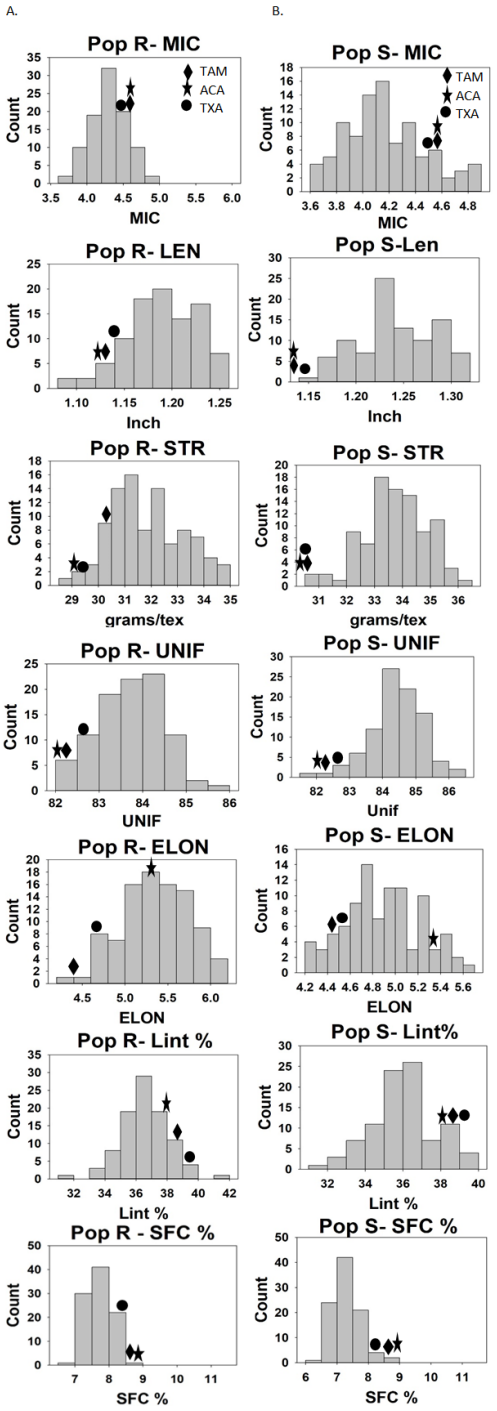

Figure 3.1- Distribution of genotypes in populations for different fiber traits. (A) pop R and (B)

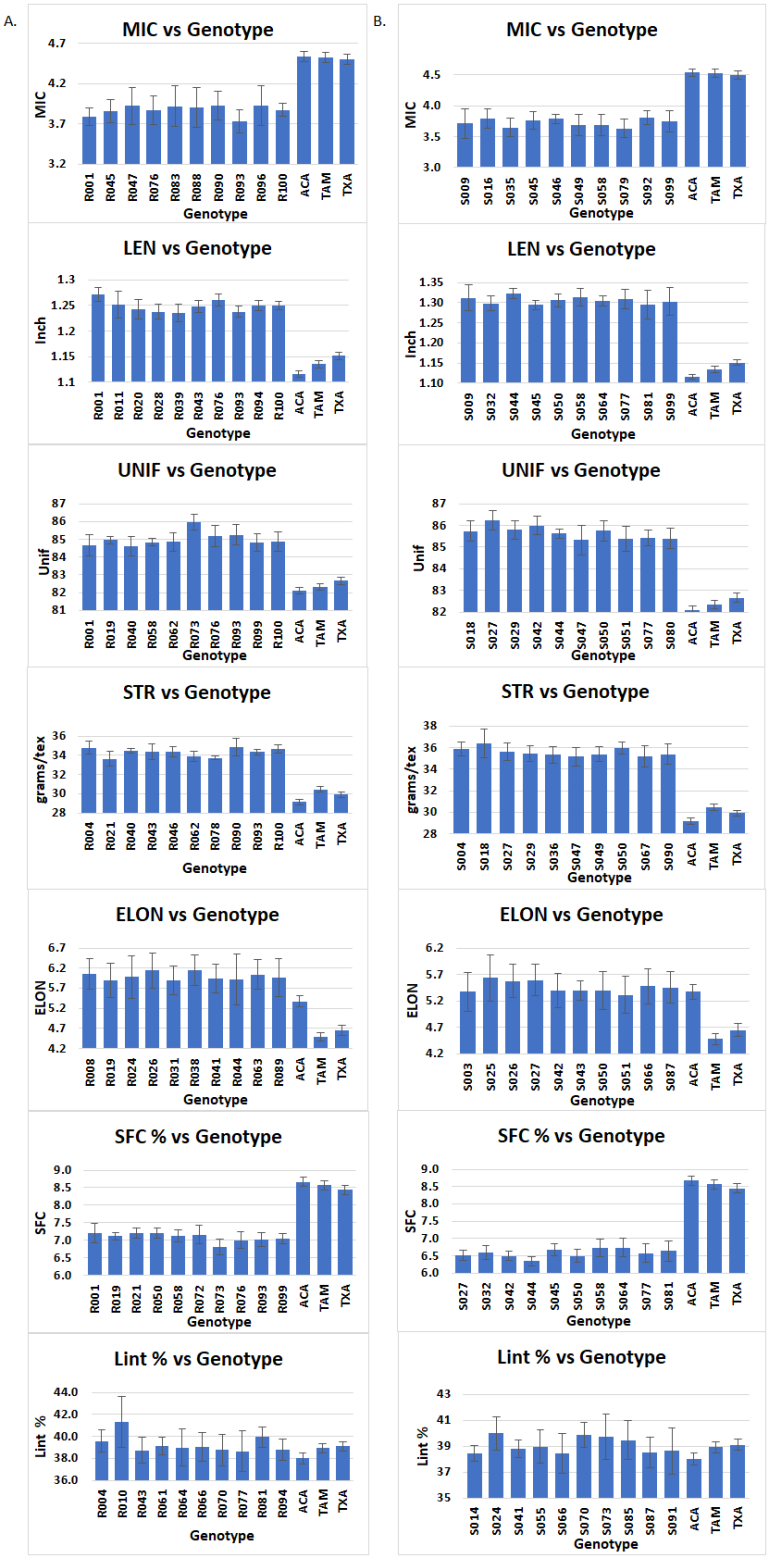

Figure 3.2- The 10 best lines for different fiber traits compared with parental lines. (A) pop R and (B) pop S

CHAPTER 4

A TERMINAL DELETION ON CHROMOSOME 18 CAUSES THE *Li*$_2$ SHORT FIBER PHENOTYPE AND EXEMPLIFIES THE COMPLEX NATURE OF COTTON FIBER QTLs

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Abstract

Extreme elongation distinguishes about one-fourth of cotton seed epidermal cells as 'lint' fibers useful for the textile industry, from 'fuzz' fibers (< 5 mm). *Ligon lintless-2* (*Li_2*), a dominant mutant with no lint fiber but normal fuzz fiber, offers insight into pathways and mechanisms that differentiate spinnable cotton from its progenitors. A population of 1,545 F2 plants derived from a cross between Pima S-7 (*G. barbadense*) and an *Li_2* mutant line (*G. hirsutum*) and 144 DNA markers were used to fine map the *Li_2* region on chromosome 18. CISP15 was 0.4 cM from *Li_2* and two 'dominant' markers (i.e. with null alleles in the *Li_2* genotype) SSR7 and SSR18 showed complete linkage with *Li_2*. Non-random distribution of markers with null alleles suggests the *Li_2* phenotype to result from a 176-221 kb deletion of the terminal region of chromosome 18 that may have been masked in prior pooled-sample mapping strategies. The deletion includes at least 40 genes, among which 10 had annotations suggesting roles in fiber development and contained a total of 13 SNP alleles differentiating non-fiber and fiber producing *Gossypium* species, with 11 in coding regions of 8 genes inferred to cause amino acid changes. Based on qPCR at seven different fiber development stages, the 10 candidate genes in the putative *Li_2* deletion showed 5 broad categories of expression patterns. Two genes with a striking expression difference during fiber elongation stages show a VIGS induced *Li_2* like phenotype, each belonging to Glycosyltransferase Family 1. However, at least 7 of the 10 genes with annotations related to fiber development showed higher expression in wild-type compared to *Li_2* mutant during fiber development stages, consistent with the hypothesis that some cotton fiber (and perhaps other) QTLs comprise groups of closely-spaced genes that are functionally diverse but coordinately regulated.

Introduction

The ability of single cotton fiber cells to reach as much as 6 cm in length makes them the longest cell known in the plant kingdom (KIM AND TRIPLETT 2001) and confers their value to the textile industry. There are four major stages of cotton fiber development, i.e. fiber cell initiation, elongation, secondary wall biosynthesis, and maturation (BASRA AND MALIK 1984) After initiation, only about one fourth of cotton seed epidermal cells elongate and differentiate into spinnable lint fibers while the remainder become shorter fuzz fiber (BASRA AND MALIK 1984; TIWARI AND WILKINS 1995; KIM AND TRIPLETT 2001). Fiber elongation typically last for 15 to 20 days, during which the fiber expands at a rate of 2mm/day (LEE *et al.* 2007b; XU *et al.* 2008). Genes participating in regulation of H_2O_2 and reactive oxygen species (ROS), Ca2+, stress, brassinosteroids, water transportation, cell wall loosening, different secondary metabolic pathways, ethylene related signaling pathways, and pectin biosynthesis have been identified (HOVAV *et al.* 2008a; PANG *et al.* 2010; HAIGLER *et al.* 2012; LI *et al.* 2013; FANG *et al.* 2014; SHAN *et al.* 2014; TANG *et al.* 2014; YANG *et al.* 2014). Secondary cell wall synthesis overlaps fiber elongation, during which large amounts of cellulose are synthesized and deposited which ultimately leads to thickening of the cell wall to ~3-4 μm (MANSOOR AND PATERSON 2012). Cell wall thickening is necessary to impart fiber strength, which is an important component of cotton fiber quality (PANG *et al.* 2010). Expression of many genes related to transcription (*GhMYB1 and GhMYB7),* thaumatin-like protein *(GbTLP1),* cellulose synthase (*GhCesA4*), species-specific expansin (*GbEXPATR*), and leucine-rich repeat protein kinase (*LRR RLK*) have been detected during secondary cell wall synthesis (MUNIS *et al.* 2010; KIM *et al.* 2011; SUN *et al.* 2015; HUANG *et al.* 2016; ISLAM *et al.* 2016; LI *et al.* 2016c; FANG *et al.* 2017). The final step of fiber development is fiber maturation, which may last until 60 DPA. During this process, there is

accumulation of minerals and decline of water potential in fiber (JOHN AND KELLER 1996; MANSOOR AND PATERSON 2012). At the end of the process, approximately 500,000 elongated lint fibers will have been produced in a single fruit containing 30 to 35 seeds (HOVAV *et al.* 2008c).

Cotton mutants containing fiber anomalies are excellent tools to help in deciphering the complex process of fiber development. Several natural and artificial cotton mutants have been discovered, genetically mapped and/or used to identify and map other genes related to fiber development (RONG *et al.* 2005; HINCHLIFFE *et al.* 2011; PATEL *et al.* 2014; JIANG *et al.* 2015; HINCHLIFFE *et al.* 2016; MA *et al.* 2016; PATEL *et al.* 2016; THYSSEN *et al.* 2016; WAN *et al.* 2016; THYSSEN *et al.* 2017) and the process has been accelerated by the recent availability of cotton genome sequences (PATERSON *et al.* 2012; WANG *et al.* 2013; LI *et al.* 2014a; LI *et al.* 2015; ZHANG *et al.* 2015). GhMML3_A12, a MYBMIXTA-like transcription factor 3 /GhMYB25- like gene was identified as the cause of a naked seed mutant (N1), with Natural Antisense Transcripts (NATs) produced due to 3' antisense promoter activity causing production of 21–22 nt small RNAs and self-cleavage of the gene (GhMML3_A12) thus producing naked seeds by inhibiting fiber initiation (WAN *et al.* 2016). Three mutants with fuzzy seeds and short lint fiber have been identified in cotton, viz. Ligon lintless-1 (Li_1) (GRIFFEE AND LIGON 1929a), Ligon lintless-2 (Li_2) (NARBUTH AND KOHEL 1990; KOHEL *et al.* 1992) and Ligon lintless-like mutant (Lix) (CAI *et al.* 2013). While Li_1 and Li_x has significantly deformed leaves and stem, Li_2 plants show normal vegetative growth. Another recessive mutant Ligon lintless-3 (*li3*), controlling lint fiber initiation was identified in a *fl* mutant population (MA *et al.* 2016). The mutant genes Li_1, Li_2, *Lix*, and *li3* map to chromosomes 22 (D4), 18 (D13), 4 (A4), and 26 (D12), respectively (RONG *et al.* 2005; CAI *et al.* 2013; MA *et al.* 2016). A single nucleotide mutation

changing an amino acid of an actin gene, GhACT_LI1, disrupts organization of F-actin and cell morphology, thus causing a dwarf vegetative habit and lintless phenotype in *Li1* (THYSSEN *et al.* 2017). *Li₂* lies at the end of chromosome 18. Rong et al. (2005) located it 0.5 cM away from a RFLP marker A1552 and 1.8 cM from two co-segregating RFLP markers, Gate4BF10 and Coau1O05. Multiple studies have revealed the genetic position, differential gene expression and metabolite changes, but not yet identifying the mutation responsible for the *Li₂* phenotype (HINCHLIFFE *et al.* 2011; GILBERT *et al.* 2013; NAOUMKINA *et al.* 2013; NAOUMKINA *et al.* 2014; THYSSEN *et al.* 2014; NAOUMKINA *et al.* 2015).

We sought to identify the *Li₂* gene, by an approach integrating positional (genetic and physical mapping) information, evolutionary information, and differential gene expression, using Virus Induced Gene Silencing (VIGS) to evaluate candidate gene(s). The identification of 7 genes in the target region that appear to function in fiber development is consistent with a recent model (Paterson et al 2012) for cotton fiber QTLs, while two genes in which fiber length could be reduced by VIGS provide new insight into the identity of *Li₂* and the functions of other genes related to elongation of the longest single cell in the plant kingdom.

Materials and Methods

Plant material and population development

A F_2 mapping population was developed by an interspecific cross between an *Li₂* mutant (*G. hirsutum*, TM-1) and Pima S6 (*G. barbadense*), to obtain rich polymorphism of DNA markers. All F1 hybrids displayed the mutant phenotype, consistent with the dominant nature of *Li₂*. F1 plants were selfed and seed were collected, obtaining and planting total of 1,545 F2 individuals in greenhouse or field for this study. Noting that the trait sometimes shows variation within a plant, multiple bolls were observed to determine the *Li₂* trait of the F_2 plants.

Genetic marker development

Initial screening and identifying linked markers near *Li₂* used available SSR (Simple Sequence Repeat) and EST (Expressed Sequence Tag) markers from multiple published genetic linkage maps (PARK *et al.* 2005; RONG *et al.* 2005; GUO *et al.* 2007; RONG *et al.* 2007; YU *et al.* 2007; ZHANG *et al.* 2008; XIAO *et al.* 2009; ZHANG *et al.* 2009; WANG *et al.* 2013). From each map, polymorphic markers near the telomere of the long arm of chromosome 18 (SHAN *et al.* 2016) were mapped with respect to *Li₂*. As markers were exhausted, closely-linked markers were used to select BACs from a *G. raimondii* library that were sequenced, developing new markers from BAC sequences using CID (http://www.shrimp.ufscar.br/cid/index.php) (FREITAS *et al.* 2008) and SSR locator (DA MAIA *et al.* 2008). We also developed CISP (Conserved Intron Scanning Polymorphic) markers as described (FELTUS *et al.* 2006). A total of 144 markers from these different approaches were first tested in a small population to check polymorphism and proximity to *Li₂*. Seven markers with clear bands and which immediately flank *Li₂* were mapped in all 1,545 F2 individuals. Genetic maps were built using JoinMap 4.1 (VAN OOIJEN 2011).

Screening of BAC library

For chromosome walking, cotton BAC libraries were screened with overgo probes designed from genetic markers mapping closely to the *Li₂* phenotype, identifying 124 BACs that might be in the *Li₂* region (Table s1). To reduce the chances of false positive hits and to minimize sequencing redundant BACs, we used a *Gossypium raimondii* physical map (LIN *et al.* 2010), designing probes from tightly linked genetic markers and hybridizing them to *Gossypium raimondii* BAC library and corresponding contigs which led to contig ctg2409 on the physical map (LIN *et al.* 2010). We sequenced three BACS namely GR174O23, GR109E22, and GR174F21, from which markers developed from GR174F21 were more tightly linked with the

Li$_2$ phenotype than the others. With the availability of the *G. raimondii* genome sequence, we BLASTed 13,662 BAC end sequences (BESs) from the *G. raimondii* library (LIN *et al.* 2010) to identify BACs with one end sequence clearly anchored to the terminal region of chromosome D 13, but the other end unanchored. The goal was to find BACs that could be sequenced to extend the terminal region of chromosome 18 and get closer to *Li*$_2$. To determine how informative these BACs will be as compared to GR174F21, we performed DNA fingerprinting.

Validation of deletion

A total of 12 genes were selected from across the target region, with two between CISP15 and the centromere and ten between CISP15 and the telomere. Primers (20-24 bp with ~500 bp amplicons) were designed using Primer 3 (http://frodo.wi.mit.edu/). All amplicons had at least 5 SNPs between the At and Dt subgenomes of tetraploid genome. Three genotypes each from *Li*$_2$ homozygous and wild type along with 12 pair of primers were amplified using the thermal profile: 95˚C for 30 sec (denaturation), 55˚C for 30 sec (annealing), 72˚C for 1 min (extension) for a total of 36 cycles, followed by a terminal extension step at 72˚C for 10 min. PCR amplicons were separated in 10% SDS-PAGE gels. Amplicons were treated with exonuclease I and shrimp alkaline phosphatase enzymatic digestion to remove unused primers and dNTPs, then amplified with BigDye mixture at 95˚C for 5 min and then 95˚C for 10 sec, 50˚C for 5 sec, and 60˚C for 4 min for a total of 30 cycles. A clean up step was performed using Sephadex plates to remove excessive primers and dyes, and sequenced on an ABI3730 capillary sequencer. Sequence analysis was done using the Bioedit program (HALL 1999) and the Codon Code Aligner software (CodonCode Corporation, Dedham, Massachusetts, USA) to check deletion of the terminal end of chromosome 18 in *Li*$_2$ mutant lines compared to wild type lines. We extracted At and Dt

sequence from the *G. hirsutum* genome using a python script to compare SNPs in the sequenced regions and to validate absence of the Dt genomic region in Li_2 mutants (LI *et al.* 2015)

Mining for putative candidate gene(s)

A 221 kb candidate region was extracted from the *G. raimondii* sequence (PATERSON *et al.* 2012) using a python script, and scanned using FGENESH to determine mRNA and protein sequences of putative genes (SALAMOV AND SOLOVYEV 2000) using the protein sequences of these genes for Blastp (protein-protein blast) in the NCBI protein database to identify possible homologs and deduce possible functions. A total of 10 putative candidate genes were identified in the region which might serve in fiber elongation process.

Comparing gene sequences between different cotton genomes

A and AtDt (tetraploid) genome cottons produce elongated fibers whereas D and F genome cottons (APPLEQUIST *et al.* 2001; HOVAV *et al.* 2008a; CHAUDHARY *et al.* 2009a) do not. As reference sequence data from the D genome (*G. raimondii*) and resequencing data of the A (*G. herbaceum* L.), F (*Gossypium longicalyx*) and AtDt (*G. hirsutum* 'Acala Maxxa', *G. hirsutum* race yucatanense, and *G. mustelinum*) genomes were available (PATERSON *et al.* 2012), sequences of predicted genes were compared by using Burrows-Wheeler Aligner (BWA) (LI AND DURBIN 2009) to align resequencing data to the reference *G. raimondii* (PATERSON *et al.* 2012) and Sequence Alignment/Map (SAMtools) (LI *et al.* 2009) was used for SNP calling.

RNA isolation and quantitative reverse transcription-PCR

Different stages of cotton boll development i.e. fibers at initiation, elongation, and secondary cell wall thickening stages were used to examine the expression profiles of 10 genes using qRT-PCR. Tissue samples were collected at 3 (fiber + ovule mix), 6 (fiber +ovule mix), 10 (fiber), 12 (fiber), 15 (fiber), 21 (fiber) and 24 (fiber) days post anthesis (DPA). RNA was extracted using

the Purelink Plant RNA reagent kit, according to the manufacturer's instructions (Life Technologies Corporation, Grand Island, NY) with a DNA digestion step using turbo DNA-free kit (Applied Biosystems, Waltham, Massachusetts, USA). Quality of RNA was determined by electrophoresis in 1% agarose gels and concentration was determined in a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). A total of 500 ng of mRNA was used to obtain first-strand cDNA using a SuperScript™ III Reverse Transcriptase cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR was carried out on a Roche LightCycler480 II instrument (Roche Diagnostics Ltd, Rotkreuz, Switzerland) in a 25-µl volume containing 10 ng of cDNA, 5 pM of each primer, and 25 µl of Fast SYBR Green Master Mixture (Thermo Fisher Scientific) according to the manufacturer's protocol. The PCR conditions were as follows: primary denaturation at 95 °C for 20 s followed by 40 amplification cycles of 3 s at 95 °C, and 30 s at 60 °C. Melting curve analysis was performed to ensure there was no primer-dimer formation. Three replicate assays were performed with independently isolated RNAs, and each PCR reaction was loaded in triplicate. Relative expression levels of each gene are presented using the $2^{-\Delta Ct}$ method (Livak *et al.*, 2001). The cotton *GhACTIN1*gene was used as the positive control.

Virus-induced gene silencing (VIGS) assay

Through PCR, from cDNA we amplified 351 to 599 bp fragments for all 10 putative candidate genes in the suspected deleted part of chromosome 18. These fragments were cloned and inserted into the VIGS binary vector pYL156 (pTRV2) that was double digested with either EcoRI KpnI, EcoRI XhoI or KpnI XhoI. The vectors pTRV1(pYL192) which is a helper plasmid, and cloned pTRV2 vectors were introduced into *Agrobacterium* strain GV3101 through electroporation (Bio-Rad Gene Pulser II). For the VIGS assay, the transformed *Agrobacterium*

colonies containing pTRV1 and cloned pTRV2 were inoculated into 5 mL of LB medium containing kanamycin (50 μg/mL) and gentamycin (25 μg/mL) and grown overnight at 28 °C in an Innova 4080 shaking at a speed of 80 rpm. The next day, the cultures were transferred into a flask containing 50 ml LB media with kanamycin (50 μg/mL) and gentamycin (25 μg/mL) antibiotic concentration plus 10 mM MES (2-(4 morpholino)-ethane sulfonic acid) and 20 μM acetosyringone and left to grow overnight at 28 °C with shaking at 80 rpm (GAO *et al.* 2011). The culture was resuspended using infiltration buffer (10 mM MgCl2, 10 mM MES and 200 μM acetosyringone). Final OD 600 of the culture was adjusted to 1.5. Seven-day old seedlings with fully expanded cotyledons but no visible true leaf were infiltrated with solution made from mixtures of pTRV1 and pTRV2 vectors in ratio of 1:1. Infected plants along with controls were grown in growth chambers under 15 hour light/ 9 hour dark cycles with light intensity of 120 μE m^{-2} S^{-1} and temperature of 24 °C.

Results

Phenotyping of *Li₂* and segregation of the *Li₂* region

The *Li₂* mutant has seeds with no lint fiber but normal fuzz fiber. To determine whether a plant was *Li₂* or wild type, multiple open bolls were inspected on each plant (Figure 1). Some plants showed both *Li₂* and wild type bolls (Figure 1), in which case the plant was considered *Li₂*. Much literature has shown the *Li₂* mutant phenotype to be genetically dominant and governed by a single locus, thus a segregation ratio of 3 lintless to 1 linted individual(s) was expected (NARBUTH AND KOHEL 1990; KOHEL *et al.* 1992; RONG *et al.* 2005). However, among 1,545 F2 plants we found 1091 to be lintless and 454 wild type, a 2.4:1 ratio suggesting enrichment of the wild type. As shown in Table 1, a similar pattern was also observed for DNA marker genotypes

in the *Li₂* region. Such segregation distortion may be due to presence of favorable alleles in this region from *G. barbadense*, or to a deleterious effect of the *Li₂* mutant (from *G. hirsutum*).

Fine mapping of the *Li₂* locus

Initial mapping of the *Li₂* locus in 135 individuals showed 12 markers to all be toward the centromeric side of the gene, i.e., lacking flanking markers on the telomeric side that would permit identification of recombinants in the interval containing the gene. To try to develop markers on the telomeric side of *Li₂*, we designed overgo probes using genetic sequences of markers closest to the gene (Gr_ea17f11, A1552, Gh.fbr.sw02661 and COAU2K07), hybridized them to *Gossypium raimondii* BACs, and sequenced the BACs, namely GR174F21, GR109E22, and GR174O23 (figure S1A). Based on alignment and segregation of markers developed from these BACs with *Li₂*, it appeared that GR109E22 and GR174O23 overlapped each other, but there was a gap between GR109E22 and GR174F21, with the latter being closest to *Li₂*. Also, no new BACs were identified that were distal to GR174F21, suggesting that it is at the end of the chromosome. With the availability of the *G. raimondii* sequence (Paterson et al. 2012), the gap between GR174F21 and GR109E22 was determined to be 26.7 kb. The remaining sequence at the terminal end of chromosome D13 (chromosome 18 in *G. hirsutum*) from GR174F21 was determined to be 37.6 kb, including 14.5 kb of undefined base pairs (N instead of A, C, G and T). We BLASTed 13,662 BAC end sequences from the *G. raimondii* library (LIN *et al.* 2010) to seek BACs with one end in the terminal region of chromosome D13, finding GR102N11 and GR006L12, but DNA fingerprinting showed that sequencing these BACs would not improve coverage of the region (figure 1B). GR174F21 was later found to extend from 60,413,412 to 60,534,221bp of chromosome D13 of *G. hirsutum*, with just 77 bp beyond it to the end of the

chromosome (ZHANG *et al.* 2015). Thus, GR174F21 was the last informative BAC sequence for the terminal region of chromosome D13.

For fine mapping, four markers NAU2980, NAU3447, NAU3827 and NAU3223 covering the *Li₂* region were used along with new markers developed from BAC GR109E22 (CISP15) and GR174F21 (SSR 7 and SSR18). A total of 1,545 F2 plants were genotyped. Based on the genetic map CISP15 was 0.4 cM away from *Li₂* while SSR7 and SSR18 co-segregated with *Li₂* (figure 2). Both co-segregating markers were dominant and in repulsion phase with *Li₂*, somewhat limiting their value.

Deletion of a chromosome segment appears responsible for the *Li₂* phenotype

To try to mitigate the constraint that SSR7 and SSR18 from BAC GR174F21 were genetically dominant, two more SSRs (CISP39 and gr174F21_4.4) were found to be polymorphic and map near *Li₂* and previously identified SSRs. However, while the majority of SSRs in other regions had been co-dominant, these (like SSR7 and 18) were also dominant, all four only amplifying *G. barbadense* alleles. This observation suggested the hypothesis that a segmental deletion near the chromosome 18 terminus may account for the *Li₂* mutant, with its genetic dominance due to haploinsufficiency, i.e. a single functional allele does not produce enough gene product to confer a wild-type phenotype. To further investigate the hypothesis of a deletion in the *Li₂* region of chromosome 18, we sequenced additional segments in this region from the wild type (*G. barbadense)* and homozygous *Li₂* mutants (*G. hirsutum*). The D-genome sequence was mined for genic sequences beyond the last co-dominant marker, i.e. CISP15 towards the telomere. Although precautions were taken while designing primers and running PCR, only 5 gene primer sets amplified single bands useful for further study. One (Gorai.013G268200) was between CISP15 and the centromere; and four, namely

Gorai.013G269200, Gorai.013G270000, Gorai.013G270800, and Gorai.013G271400 were between CISP15 and the telomere. As *Li₂* lies on a chromosome of the Dt genome, amplicon sequence in the hypothetical deletion region from *Li₂* homozygous mutants should only show sequence from the At genome while wild type plants should show sequences from both At and Dt genomes. Sequencing of Gorai.013G268200 which is between the centromere and co-dominant marker CISP 15, suggested presence of both genome (At and Dt) segments in mutant and wild type. Furthermore, due to stringency of the primers of Gorai.013G269200 only Dt was amplified in *Li₂* and wild type, suggesting no deletion in *Li₂* mutant till this point. Amplicons of three genes, Gorai.013G270000, Gorai.013G270800, and Gorai.013G271400 were missing Dt segments in *Li₂* homozygous lines, with only genotypes corresponding to the At genome present in the mutants whereas both At and Dt genotypes were present in the wild type (Figure 2). Thus, three consecutive primer pairs fail to amplify a chromosome 18 (Dt) locus from the *G. hirsutum* genotype carrying *Li₂*, while Gorai.013G268200 and Gorai.013G269200 (closer to CISP15 than the other three) amplify both At and Dt loci, supporting the hypothesis that a terminal deletion is responsible for the *Li₂* phenotype. The results suggest the deletion breakpoint to be between Gorai.013G269200 and Gorai.013G270000, with at least 176 kb and perhaps as much as 221 kb missing from the terminal end of chromosome D13 in *Li₂* mutants.

Candidate *Li₂* gene sequences

The putative 176-221 kb deletion of chromosome 18 in the *Li₂* mutants spanned a total of 40 genes, among which 10 had annotations suggesting function as cell wall proteins and cell wall enzymes, or associated with secondary metabolism, hormonal regulation, and post-transcriptional modification. To further narrow down the list we compared coding sequences (CDS) and untranslated regions (5’ and 3’ UTRs) of the 10 genes between non-fiber producing

(D and F genomes) and fiber producing (A, At, and Dt genome) taxa. We found 13 SNP alleles differentiating non-fiber and fiber producing groups, with 11 in coding regions of 8 genes inferred to cause amino acid changes and potentially affect gene function (Table 2).

Differential expression of candidate gene during fiber development based on Rt-qPCR

Based on qPCR at seven different fiber development stages (Figure 4), the 10 candidate genes in the putative *Li₂* deletion showed 5 broad categories of expression patterns. Two genes (*GhUBE11-D1a* and *GhC4H*) were both expressed primarily at 3 and 6 DPA, with substantially diminished expression later, and with only small differences between *Li₂* and wild type. Four genes (*GhUGT87A1-D1a*, *GhUGT87A2*, *GhUGT87A1-D1b*, *GhETO1*) were expressed at low levels at 3 DPA, with much higher levels at 6 and 12 DPA (and higher in wild type than *Li₂*), diminishing at 15 DPA with wild type maintaining higher expression than *Li₂* for *GhUGT87A1-D1a* and *GhETO1* (and noting that the transition from 6 to 10 DPA is confounded with sampling of ovule versus fiber tissue so is not directly comparable). Two genes (*GhE1310*, *GhEXPA8*) paralleled the general expression pattern of the second group, but with striking enrichment of expression in *Li₂* at 15 DPA. *GhIRX7* showed *Li₂* enriched expression early in development (3-6 DPA) but wild-type enriched expression late (21, 24 DPA). *GhUBE11-D1b*, a ubiquitin-activating enzyme, was enriched in wild-type at all developmental stages except 10 DPA.

Virus-Induced Gene Silencing (VIGS)

To further investigate the 10 candidate genes in the putative *Li₂* deletion, we cloned fragments of each gene, ranging in size from 351 to 599 bp. Due to constraints on growth chamber space, we performed an initial VIGS experiment in the green house with monitored temperature and humidity, finding significant reduction in fiber length of plants treated with TRV2:UGT87A1-D1a and TRV2:UGT87A2. In a follow up study in a growth chamber, multiple plants treated

with TRV2:UGT87A1-D1a showed 32 to 40% reduction in fiber length compared to control lines, with 17 to 23% reduction in fiber length for plants treated with TRV2:UGT87A2 (Figure 5). Despite the use of a growth chamber with controlled environmental conditions validated in prior work (GAO *et al.* 2011), VIGS phenotypes varied somewhat between different plants and within the same plants, as has been reported previously in cotton (WAN *et al.* 2016; ANDRES *et al.* 2017). Similar problems were also seen in plants treated with the control vector TRV2:GRCLA1.

Phylogenetic analysis

Sequences of *UGT87A1* and *UGT87A2* genes were extracted from genome sequences of *G. raimondii* (PATERSON *et al.* 2012), *G. arboreum* (LI *et al.* 2014a), *G. hirsutum* (LI *et al.* 2015) and resequencing data for *G. longicalyx* (PATERSON *et al.* 2012). Protein sequences were predicted using FGENESH and employed in phylogenetic analysis performed using MrBayes v3.2 (KUMAR *et al.* 2016b) with the JTT model of protein evolution (JONES *et al.* 1992). The analysis was run for 5,000,000 generations with sample frequency every 100 generations. The resulting tree was visualized in MEGA7 (KUMAR *et al.* 2016b). *GhUGT87A1_D1a* and *GhUGT87A2* appear more closely related to each other than either is to *GhUGT87A1_D1b*. Further protein sequence alignment using MUSCLE (EDGAR 2004) showed truncation of *GhUGT87A2* in the At subgenome at the C-terminal end, with partial deletion (16 of 44 amino acid) of a highly conserved region known as PSPG (plant secondary product glycosyltransferase) (Figure 6) that plays a role in transferring sugar molecules from donor to acceptor molecules (OSMANI *et al.* 2009; TERASAKA *et al.* 2012). Thus, deletion in PSPG can affect normal transfer of sugar molecules by *GhUGT87A2* of the At subgenome, causing partial or complete loss of function.

Discussion

Investigation of the causal agent(s) of the cotton *Li₂* mutant phenotype, conferring seeds with no lint fiber but normal fuzz fiber, provides an early example in support of the hypothesis that groups of closely-spaced genes that are functionally diverse but coordinately regulated may be of central importance to cotton fiber development (PATERSON *et al.* 2012). Cotton fiber development comprises a long series of carefully coordinated processes, including initiation, elongation, secondary wall deposition and maturation to form the longest single cell known in the plant kingdom, and involves much of the transcriptome (KIM AND TRIPLETT 2001). Mutants in fiber initiation, elongation, and secondary cellulose synthesis (KIM AND TRIPLETT 2001; RONG *et al.* 2005; CAI *et al.* 2013) provide excellent tools for dissecting these processes, with both fundamental and commercial importance.

Two prior studies had mapped *Li₂* to a different position than ours. Hinchliffe *et al.* (2011) mapped the locus between marker DPL0547 and DPL0922, with EST-derived SSR NAU3991 completely linked with the *Li₂* locus. Based on mapping and differential gene expression data, they suggested that a putative gene producing plectin-related protein might be responsible for the *Li₂* phenotype. Using 'super bulked segregant analysis' sequencing (sBSAseq) and a larger F2 population of the same pedigree used by Hinchliffe *et al.* (2011), Thyssen et al. (2014) identified two putative *Li₂* candidate genes, TIP (aquaporin) and ZnF (C2H2-type zinc finger family protein) although finding no changes in the coding sequences of any genes in the *Li₂* vicinity. Based on a *G. hirsutum* sequence (ZHANG *et al.* 2015), the *Li₂* candidate (Gh_D13G2394) identified by Thyssen et al. (2014) was between and nearly equidistant from our markers CISP15 (260.7 kb) and NAU2980 (270 kb). In our study, recombination between these markers, additional markers in the deletion region and the *Li₂* phenotype placed the *Li₂* gene in the

deletion region rather than the region suggested by Thyssen et al. (2014). We found a total of 22 recombinations between these markers and *Li₂* always segregated with marker CISP 15, a finding that would be extremely improbable if the *Li₂* gene was where Thyssen et al. (2014) suggest.

The strategy used by Thyssen et al. (2014) of bulking 100 segregants based on *Li₂* phenotype, could mix *Li₂* homozygotes and heterozygotes, masking SNPs in the deletion region that we suggest to be critical to the *Li₂* phenotype since heterozygotes will have at least one wild type allele. Also, diploid *G. raimondii* was the best available reference genome when they conducted their study, which might have differed somewhat from the tetraploid genome and diminished the accuracy of mapping the region. A similar complication was observed in mapping the *Li1* gene using diploid *G. arboreum* and *G. raimondii* for aligning SNPs (THYSSEN *et al.* 2015; THYSSEN *et al.* 2017). While two nearby regions of a chromosome could each include different genes conferring a similar phenotype, for example seed shattering in sorghum involving *SpWRKY* and *YABBY* loci which are only 300 kb apart (LIN *et al.* 2012; TANG *et al.* 2013), this scenario is rare.

Haploinsufficiency, such as we postulate to account for the genetic dominance of the mutant *Li₂* allele, has been reported in plants, albeit rarely (MEINKE 2013; YUAN *et al.* 2014a). Our observations corroborate prior reports of abnormal expression of the *Li₂* phenotype and incomplete penetrance of the *Li₂* gene (AN *et al.* 2010), resembling haploinsufficiency and incomplete penetrance associated with segmental deletion in multiple human studies (KÖHN *et al.* 2009; KLAASSEN *et al.* 2013; TODARELLO *et al.* 2014; EL KHATTABI *et al.* 2015; KLAASSEN *et al.* 2016). The scarcity of haploinsufficiency in plants might be due to a greater degree of tolerance of plant genomes than others for changes of gene dosage, as plant lineages have incurred genome duplications more frequently than any other taxa known (PATERSON *et al.*

2010). Genes with similar function or connected genes tend to cluster in genomes (NEI 2003; YI *et al.* 2007). Deletion of part of a chromosome containing connected genes might have a haploinsufficient effect as one copy of multiple genes involved in a biosynthetic pathway is removed, causing disruption in the process (THOMAS *et al.* 2006). QTL clusters for single or multiple fiber traits have been identified in cotton (RONG *et al.* 2007; LI *et al.* 2016a; ZHANG *et al.* 2016b) and related to closely-spaced genes that are functionally diverse but coordinately regulated (PATERSON *et al.* 2012).

The failure of fibers to elongate properly in the absence of two wild-type Li_2 alleles suggests that the Dt genome of tetraploid cotton may be differentiated from its diploid progenitor by a neomorphic mutation. While diploid D-genome cottons do not produce lint fibers, QTL studies have shown many fiber-quality related QTLs to locate in Dt genomic regions of tetraploid cottons (RONG *et al.* 2007; ZHAO *et al.* 2012; WANG *et al.* 2013). The fact that loss of even a single wild-type Li_2 allele causes extremely short fiber suggests that homologs of these genes, including homoeologs in the At genome, are functionally diverged and not able to compensate for this loss. Having a partially functional homologous gene/s may cause abnormal expression of the wild phenotype and incomplete penetrance such as we observe. A careful study of gene expression using RNAseq in plants with such atypical phenotypes might clarify such an anomaly.

Based on gene expression and comparative genomics results, we found seven genes namely, *GhIRX7_D*, *GhETO1_D*, *GhUBE11_Db*, *GhUGT87A1_Da*, *GhUGT87A2_D*, *GhUGT87A1_Db* and *GhEXPA8* that might have roles in fiber development. Employing VIGS, we could see impact of knocking down of genes *GhUGT87A1_Da* and *GhUGT87A2_D* on fiber length, confirming their importance in fiber elongation.

GhIRX7_D belongs to the glycosyltransferase family 47 (ZHONG AND YE 2003; GESHI *et al.* 2010). Disrupting of *IRX7* or *FRA8 (FRAGILE FIBER 8)* causes reduction in secondary wall thickness, decline in amounts of cellulose and xylan, collapse of xylem vessels, decrease in stem strength, and dwarf phenotype (BROWN *et al.* 2005; ZHONG *et al.* 2005; BROWN *et al.* 2007; LEE *et al.* 2007a). Reduced expression during secondary wall synthesis (Figure 4) of *GhIRX7_D* in *Li*$_2$ mutants might also affect secondary wall biosynthesis, contributing to the phenotype at later stages of fiber development.

GhEXPA8 belongs to the expansin superfamily, wall-loosening proteins which help with cell expansion (SAMPEDRO AND COSGROVE 2005). Multiple studies have discovered the role of expansin genes during fiber development, especially during fiber elongation (ORFORD AND TIMMIS 1998; JI *et al.* 2003; SHI *et al.* 2006; SHAN *et al.* 2014; BAJWA *et al.* 2015). The result suggests that *GhEXPA8* is important for fiber length and any reduction in its expression during fiber elongation (as seen in *Li*$_2$ mutant) might have repercussions.

GhETO1_D encodes an ethylene-overproduction protein. In Arabidopsis, *ETO1* controls the rate of ethylene synthesis by negatively regulating 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (WANG *et al.* 2004; YOSHIDA *et al.* 2005; CHRISTIANS *et al.* 2009)**.** Ethylene plays an important role in fiber elongation but excessive production might curb fiber development (SHI *et al.* 2006). A steep increase of tenfold in ethylene concentration is seen in mutants with loss-of-function *eto1* mutations causing shorter seedlings, smaller leaves, and reduced sizes of roots, petioles and inflorescences (CHAE *et al.* 2003; WANG *et al.* 2004; CHRISTIANS *et al.* 2009; PANG *et al.* 2010; LUO *et al.* 2014)**.** Application of abscisic acid (ABA) enormously inhibited root growth in *eto1* mutants by promoting ethylene biosynthesis (LUO *et al.* 2014). Elevated levels of ABA have been observed during fiber development of *Li*$_2$ mutant

lines, which coupled with reduced expression of *GhETO1_D* during fiber elongation might have contributed to the *Li₂* phenotype (CHEN *et al.* 1997; GILBERT *et al.* 2013). *GhUBE11_Db* encodes Ubiquitin-activating enzyme E1 that catalyzes the first step of three consecutive enzymatic cascades in a ubiquitination reaction. Interestingly, regulation of ethylene biosynthesis through ubiquitin-mediated protein degradation is assisted by ethylene-overproduction protein (ETO1) (LYZENGA AND STONE 2012). This suggests that multiple genes in the same biological process have been affected by deletion in *Li₂* mutants.

GhUGT87A1_Da, *GhUGT87A2_D* and *GhUGT87A1_Db* belong to Glycosyltransferase (GT) Family 1, the largest GT family (YONEKURA-SAKAKIBARA AND HANADA 2011; HUANG *et al.* 2015). *UGT87A1* and *UGT87A2* are nearly identical, and may have similar function and redundant effects. *UGT87A2* in *Arabidopsis* has been involved in ascorbic acid homeostasis, cell wall biosynthesis, controlling H_2O_2 level (ROS scavenging activity), delaying flowering, enhancing germination, root growth and prevention of cell damage during stress (SAINT PAUL 2010; WANG *et al.* 2012; LI *et al.* 2016b). Regulation of H_2O_2 or ROS is required to avoid arresting the fiber elongation process, as higher levels of H_2O_2 could trigger secondary cell wall biosynthesis (LI *et al.* 2007; CHAUDHARY *et al.* 2008; HOVAV *et al.* 2008a; CHAUDHARY *et al.* 2009a; GUO *et al.* 2016). Hovav *et al.* (2008) found elevated concentration of H_2O_2 in *G. herbaceum* (A genome) and *G. longicalyx* (F genome) during early fiber elongation, but the latter was not able to reduce the H_2O_2 level thus reducing fiber elongation, enhancing stress conditions and triggering secondary cell wall biosynthesis. Deletion of these genes might have increased H_2O_2 or ROS accumulation, permitting premature halting of fiber elongation and causing the *Li₂* phenotype. Such higher concentration of ROS and higher expression of stress related genes have been reported in *Li₂* mutant (HINCHLIFFE *et al.* 2011; NAOUMKINA *et al.* 2013). Further, better

germination was observed in *87A2OE* than *ugt87a2* knock out mutant line and wild type (LI *et al.* 2016b). We also observed segregation distortion in our population with significant reduction of plants having homozygous *G. hirsutum* alleles (homozygous Li_2 mutant allele), suggesting some role of these genes in reproductive organs, seed viability and germination.

While the cotton Li_2 mutant phenotype is essentially discrete and tentatively due to two specific genes, we posit that the putative deletion conferring this phenotype may exemplify consequences of allele substitution at many cotton (and perhaps other) QTLs, comprising groups of closely-spaced genes that are functionally diverse but coordinately regulated (PATERSON *et al.* 2012).

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Table 4.1 Segregation distortion of DNA markers in the ***Li₂*** region

	CISP15			**NAU2980**			**NAU3827**		
Genotype	observed	expected	Chi test	observed	expected	Chi test	observed	Expected	Chi test
GH/GH	284	380.00	2.02E-08	285	382.75	2.16E-08	288	383.50	7.56E-08
GH/GB	796	780.00		808	765.50		814	767.00	
GB/GB	440	380.00		438	382.75		432	383.50	
Total	1,520			1,531			1,534		

Table 4.2 – Properties of non-synonymous SNPs differentiating fiber and non-fiber producing *Gossypium* species

GENE ID (*G. ramondii*)	POSITION	STRAND	Non-Fiber	Fiber	SNP location	Non-Fiber aa	Fiber aa	GENE ID (*G. hirsutum*)	Annotated function
Gorai.013G269400	58104353	-	A	T	CDS	F	Y	Gh_D13G2434	glucuronoxylan glucuronosyltransferase IRX7
Gorai.013G270200	58142691	+	G	A	CDS	G	R	Gh_D13G2442	ethylene-overproduction protein 1-like
Gorai.013G270200	58143864	+	C	A	CDS	L	I	Gh_D13G2442	ethylene-overproduction protein 1-like
Gorai.013G270200	58144018	+	G	A	CDS	G	D	Gh_D13G2442	ethylene-overproduction protein 1-like
Gorai.013G270200	58144068	+	G	A	CDS	V	I	Gh_D13G2442	ethylene-overproduction protein 1-like
Gorai.013G270300	58144807	-	A	G	3-UTR	.	.	Gh_D13G2443	ubiquitin-activating enzyme E1 1-like
Gorai.013G270300	58144988	-	T	G	3-UTR	.	.	Gh_D13G2443	ubiquitin-activating enzyme E1 1-like
Gorai.013G270300	58145428	-	A	G	CDS	L	L	Gh_D13G2443	ubiquitin-activating enzyme E1 1-like
Gorai.013G270400	58154218	-	T	A	CDS	Q	H	Gh_D13G2444	ubiquitin-activating enzyme E1 1-like, transcript variant X2
Gorai.013G270800	58179089	+	A	G	CDS	E	G	Gh_D13G2448	glucan endo-1,3-beta-glucosidase 9-like
Gorai.013G270900	58181681	+	C	T	CDS	P	L	Gh_D13G2449	UDP-glycosyltransferase 87A1-like
Gorai.013G271000	58184415	+	C	G	CDS	R	G	Gh_D13G2450	UDP-glycosyltransferase 87A2-like, transcript variant X2
Gorai.013G271000	58185533	+	C	T	3-UTR	.	.	Gh_D13G2450	UDP-glycosyltransferase 87A2-like, transcript variant X2
Gorai.013G271100	58187286	+	T	A	CDS	I	N	Gh_D13G2452	UDP-glycosyltransferase 87A1-like
Gorai.013G271700	58235904	+	C	T	5-UTR	.	.	Gh_D13G2458	trans-cinnamate 4-monooxygenase-like
Gorai.013G272000	58243663	+	A	G	CDS	R	G	Gh_D13G2460	expansin-A8-like

a. b. c.

d.

Figure 4.1- Phenotype of seeds containing the *Li₂* mutant allele, and homozygous WT allele (a) the *Li₂* mutant allele (b) homozygous WT allele (c) a branch showing atypical expression of the *Li₂* phenotype (two bolls of *Li₂* phenotype on side with the center with normal lint or wild type phenotype); and (d) atypical expression of *Li₂* phenotype within a boll. The extreme shows normal expression of *Li₂* or wild type phenotype in a boll while central two shows abnormal phenotype in a boll of *Li₂* plant

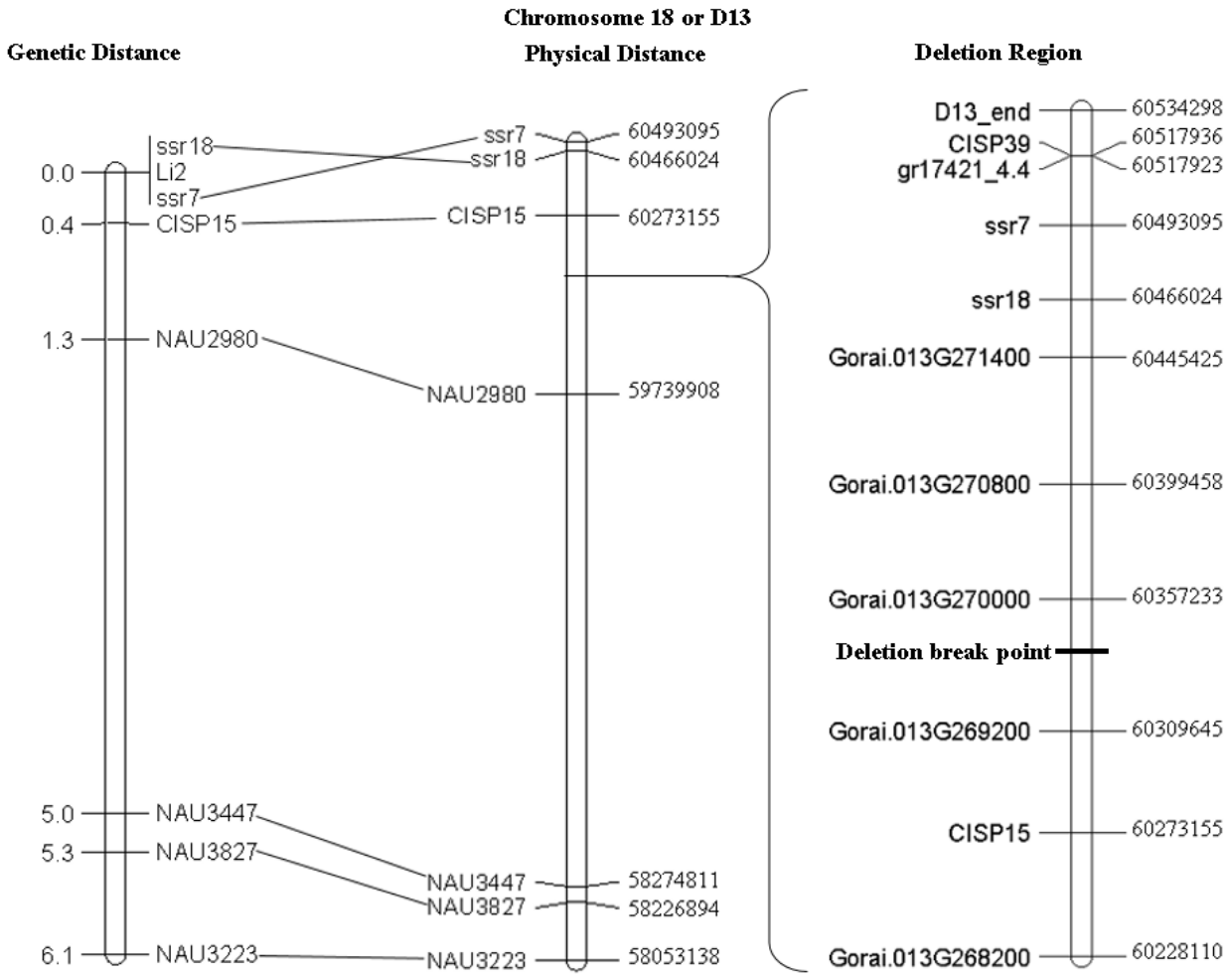
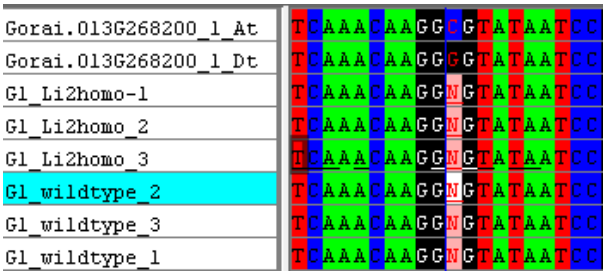
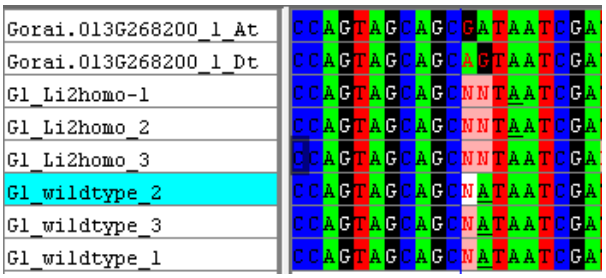


Figure 4.2- Genetic and physical map of the *Li₂* locus
Genetic distance is in cM and physical distance is in bp. An approximate deletion break point is shown. The physical map indicates that the markers from the deletion region (ssr7 and ssr18) are completely linked with the *Li₂* phenotype. Gorai.013Gxxxxxx represents genes from which amplicons were sequenced to investigate the deletion theory and find approximate break points.

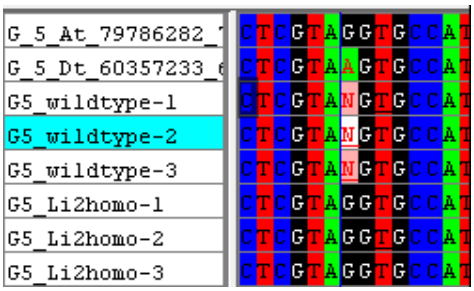
a.



b.



c.



d.

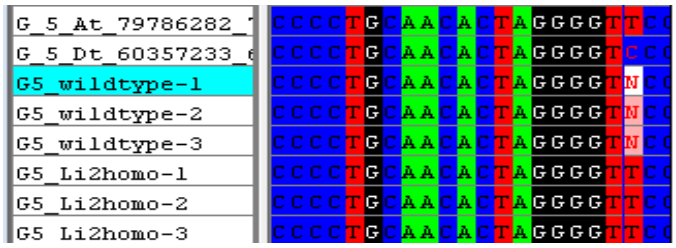


Figure 4.3 -Validating deletion of the terminal end of chromosome 18 in *Li₂* homozygous
Eight sequences are shown in these figures. The sequences are At subgenome, Dt subgenome, three biological replicates of wild type and three biological replicates of *Li₂* homozygous lines.
(a) and (b) are representation of the sequence form Gorai.013G268200. We can see both alleles from At and Dt sub genome are present in wild type and *Li₂* homozygous lines.
(c) and (d) are representation of the sequences from Gorai.013G270000. Here we can see both alleles from At and Dt sub genome are present in wild type and but allele from Dt subgenome is *Li₂* homozygous lines.

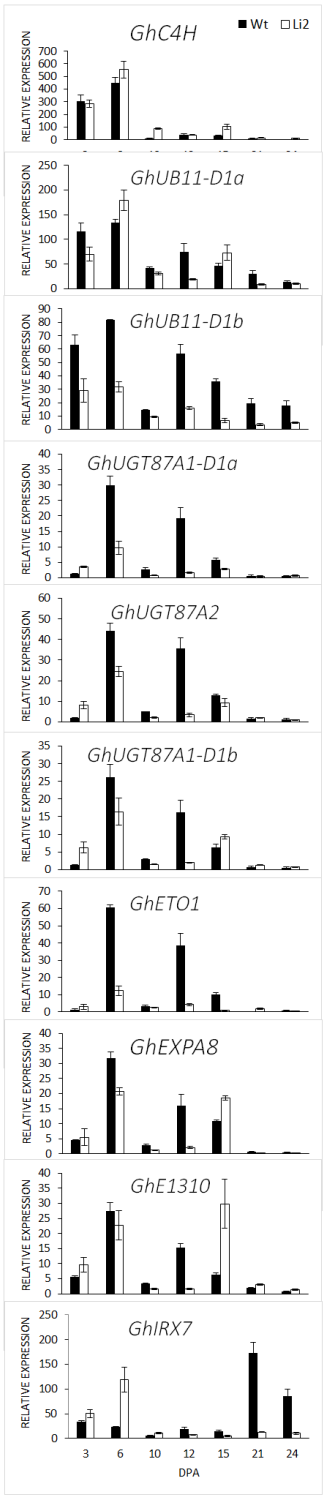
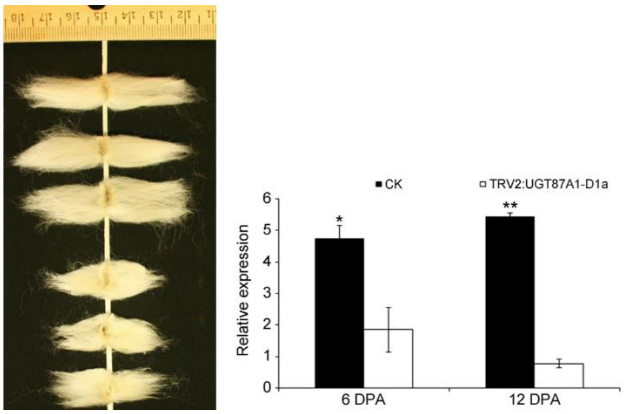
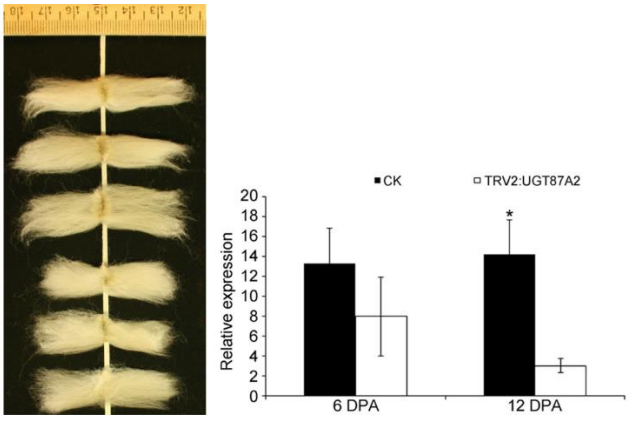


Figure 4.4 – Gene expression analysis using RT-qPCR for candidate genes in fiber tissues from wild-type and mutant plants. X-axis represents Days post-anthesis (DPA). Fiber and ovule tissues were mixed for 3 and 6 DPA, for remaining DPA (10,12,15,21 and 24) only fiber tissues were used for the study. Error bars represents standard error (SE) from three biological replicates.

a.



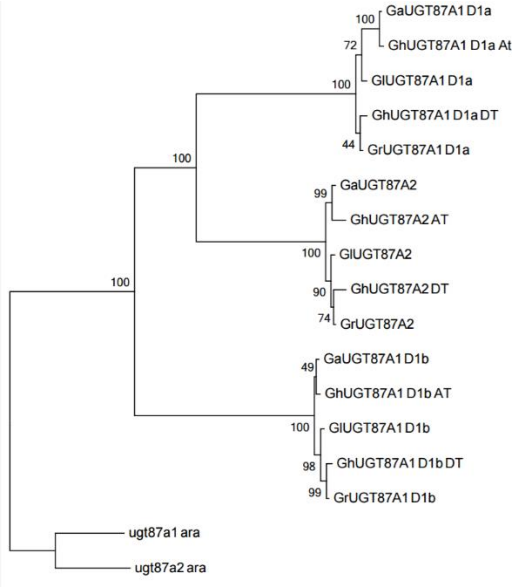
b.



c.

Figure 4.5 – Reduction of fiber length by Virus Induced Gene Silencing (VIGS)
(a) Representative plants treated with TRV2:GRCLA1 as a visual marker for verifying efficiency of viral infection. Fiber samples from plants infected by TRV2:UGT87A1-D1a (b) and TRV2:UGT87A2 (c) and relative expression of the genes at different fiber development stages (6 and 12 DPA)

a.



b.

```
ugt87a2_ara          QGSFLSVSEAQMEEIVKGLRESGVRFLWVARGGELKLKEALEGSLGVVVSWCDQLRVLCH
GaUGT87A1-D1b        MGSFLSVSNVQTEEITAGLQDSGVRFLLVAREGSWKLKHG-CGGEGLVVEWCDQLRVLCH
GhUGT87A1-D1b_AT     MGSFLSVSNVQMEEITAGLQDSGVRFLLVAREGSWKLKHG-YGGEGLVVEWCDQLRVLCH
GlUGT87A1-D1b        MGSFLSVSNVQMEEITAGLQDSGVRFLLVAREGSWKLKHG-CGGEGLVVEWCDQLRVLCH
GhUGT87A1-D1b_DT     MGSFLSVSNVQMEEITAGLQDSGVRFLLVAREGSWKLKHG-YGGEGLVVEWCDQLRVLCH
GrUGT87A1-D1b        MGSFLSVSNVQMEEITAGLQDSGVRFLLVAREGSWKLKHG-YGGEGLVVEWCDQLRVLCH
GhUGT87A2_DT         LGSFLSVSNTQMDEIVAGLQISGVRYLWVARGEASRLKDR-CGDMGLVVEWCDQLKVLCH
GrUGT87A2            LGSFLSVSNTQMDEIVAGLQISGVRYLWVARGEASRLKDR-CGDMGLVVEWCDQLKVLCH
GlUGT87A2            LGSFLSVSNTQMDEIVAGLQISGVRYLWVARGEASRLKDR-CGDMGLVVEWCDQLKVLCH
GhUGT87A2_AT         LGSFLSVSNTQMDEIVAGLQISGVRYLWVARGEASRLKDR-CGDMGLVVEWCDQLKVLCH
GaUGT87A2            LGSFLSVSNTQMDEIVGGLQISGVRYLWVARGEASRLKDR-CGDMGLVVEWCDQLKVLCH
GaUGT87A1-D1a        LGSFLSVSNDQMDEIAAGLQDSGVPYLWVARGETSWPTEK-GSEMGFVVEWCDQLKVLCY
GhUGT87A1-D1a_At     LGSFLSVSNDQMDEIAAGLQDSGVPYLWVARGETSWPTEK-GSEMGFVVEWCDQLKVLCY
GlUGT87A1-D1a        LGSFLSVSNDQMDEIAAGLQDSGVPYLWVARGGTSRPTEK-GGEMGLVVEWCDQLKVLCH
GhUGT87A1-D1a_DT     LGSFLSVSNDQMDEIAAGLQDSGVLYLWVARGGTSRLTEK-GSEMGLVVEWCDQLKVLCH
GrUGT87A1-D1a        LGSFLSVSNDQMDEIAAGLQDSGVLYLWVARGGTSRLTEK-GSEMGLVVEWCDQLKVLCH
                      *******: * :**. *:. :** :: ***    . .    .  *.**.*****.***:

ugt87a1_ara          AAIGGFWTHCGYNSTLEGICSGVPLLTFPVFWDQFLNAKMIVEEWRVGMGIERKKQMELL
ugt87a2_ara          KAVGGFWTHCGFNSTLEGIYSGVPMLAFPLFWDQKLNAKMIVEDWRVGMRIERTKKNELL
GaUGT87A1-D1b        PSIGGFWSHCGWNSVKEGIFAGTPFLTFPLFADQNLNSKLIVEDWKIGWRIKKHQ----F
GhUGT87A1-D1b_AT     PSIGGFWSHCGWNSVKEGIFAGTPFLTFPLFADQNLNSKLIVEDWKIGWRTKKHQ----F
GlUGT87A1-D1b        PSIGGFWSHCGWNSVKEGIFAGTPFLTFPLFADQNLNSKLIVEDWKIGWRIKKHQ----F
GhUGT87A1-D1b_DT     PSIGGFWSHCGWNSVKEGIFAGTPFLTFPLFADQNLNSKLIVEDWKIGWRIKKHQ----F
GrUGT87A1-D1b        PSIGGFWSHCGWNSVKEGIFAGTPFLTFPLFADQNLNSKLIVEDWKIGWRIKKHQ----F
GhUGT87A2_DT         PSVGGFWTHCGWNSILEAVFAGVPMLTFPLFLDQDTNSRQIVKDWGNGWRVNDAVKAEKL
GrUGT87A2            PSVGGFWTHCGWNSILEAVFAGVPMLTFPLFLDQDTNSRQIVEDWGNGWRVNDAVKAEKL
GlUGT87A2            PSVGGFWTHCGWNSILEAVFAGVPMLTFPLFLDQDTNSRQIVEDWGNGWRVNDAVKAEKL
GhUGT87A2_AT         PSVWGFWMHCGWNSILDV----------------------RIV-----------------
GaUGT87A2            PSVGGFWTHCGWNSILEAVFAGVPMLTFPLFLDQDTNSRQIVEDWGNGWRVNDAVKAEKL
GaUGT87A1-D1a        SSIGGFLTHCGWNSILEAIYAGIPMLTFPILFDQAPNSKQIVDDWKIGWRLKEEQKDGSL
GhUGT87A1-D1a_At     SSIGGFLTHCGWNSILEAIYAGIPMLTFPILFDQAPNSKQIVDDWKIGWRLKEEQKDGSL
GlUGT87A1-D1a        SSIGGFLTHCGWNSILEAIYAGITMLTFPILFDQAPNSKQIVDDWKIGWRLKEQQKDGSL
GhUGT87A1-D1a_DT     SSIGGFLTHCGWNSILEAIYAGIPMLTFPILFDQAPNSKQIVDDWKIGWRLKEQQKDGRL
GrUGT87A1-D1a        SSIGGFLTHCGWNSILEAIYAGIPMLTFPILFDQAPNSKQIVDDWKIGWRLKEQQKDGRL
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Figure 4.6 – Phylogenetic analysis and protein sequence alignment
(a) Bayesian inference phylogenetic analysis was performed in MrBayes3.2. The consensus tree shows that *GhUGT87A1_D1a* and *GhUGT87A2* are more closely related than *GhUGT87A1*_D1b (b) Protein sequence alignment performed using MUSCLE indicates deletion of 16 amino acid in highly conserved PSPG (plant secondary product glycosyltransferase) region of 44-amino acids, shown in red boxes

Chapter 5

SUMMARY

Cotton faces two inter-related challenges -- to identify and more effectively manipulate genes participating in complex fiber development that results in the primary economic product of cotton, while broadening the genetic diversity available to select for improved fiber quality. Here we showed how mutants can be used to decipher genes involved in fiber development and increase genetic diversity in cotton.

Studies to understand genetics and identify the causal mutation of the *Li*$_2$ phenotype have been going on for almost three decades. Due to the subtelomeric location of the *Li*$_2$ locus, it was difficult to identify two flanking markers. Numerous studies on fine mapping, gene expression and metabolomics have been conducted but none had identified the causal mutation of *Li*$_2$ -- indeed, the nature of the locus shows that some studies were flawed and could not have identified the gene. Using genetic markers and a large F_2 population, we inferred that the causal mutation for the *Li*$_2$ phenotype is a terminal deletion of around 176 Kb of chromosome 18. We further identified seven candidate genes in the deletion region that could participate in fiber elongation, two of which individually show mutant phenotypes resembling *Li*$_2$.

De novo mutants are an important part of crop improvement programs, exemplified by those that were central to the "Green Revolution". Here we evaluated ten different populations derived from 12 previously-identified mutant lines affecting various fiber quality components, to see if the improvement of fiber quality was stable, transferable and whether stacking of multiple mutant alleles could improve multiple fiber quality attributes. Multiple lines within each

population were significantly improved for the fiber trait for which the population was developed. Multiple lines were also improved for multiple fiber traits for which they were crossed, suggesting allele pyramiding from mutant lines does improve multiple fiber quality attributes. Remarkably, one population developed from mutants to improve fiber length showed 75% of lines improved for four or more fiber traits and 45% improved for five or more fiber traits. Thus, improvement of one fiber trait can simultaneously improve other fiber traits as well.

The most improved lines from each population for micronaire, fiber length, fiber strength, uniformity index, fiber elongation, and lint percent were O013 (31.7%), P058 (16.1%), K92 (22.4%), Q47 (4.1%), N068 (45.8%), and O014 (13.9%), respectively. Such genotypes should be tested for stability of the phenotype and estimation of their yield potential before releasing them as new germplasm. Genotype S50 of pop S showed striking improvement of 14.1% in MIC, 13.5% in LEN, 3.8% in UNIF, 20.1% in STR, 16.1% in ELON and 23% compared to TXA but had significant lower Lint %. Such line could be directly used in a breeding program to develop fine, long and strong fiber. Genotype S32 showed improvement of 12.6% for MIC, 12.7% in LEN, 2.8% in UNIF, 17.4% in STR, 11.8% in ELON and 21.8% in SFC and no difference in Lint % with respect to TXA. Similarly, S46 showed improvement of 15.9% for MIC, 12.1% in LEN, 2.3% in UNIF, 13.2% in STR and 18% in SFC and no difference in ELON and Lint % with respect to TXA. Such lines should be tested for yield trial and could be release as germplasm with improvement in multiple fiber traits.

Each of the two studies here provide new evidence in support of the recent hypothesis that development of the cotton fiber, a complex organ that is the longest single cell known in the plant kingdom, involves groups of closely-spaced genes that are functionally diverse but coordinately regulated (PATERSON *et al.* 2012).