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Past, Present and Future Trends in Cotton Breeding

Edited by Mehboob-Ur-Rahman and Yusuf Zafar

PAST, PRESENT AND FUTURE TRENDS IN COTTON BREEDING

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Meet the editors

Dr. Mehboob-ur-Rahman (Group Leader, Plant and Molecular Breeding Labs, NIBGE, Faisalabad, Pakistan) has been involved in exploring genomes of cotton and wheat—paving the way for the development of nine cotton and one wheat varieties. His group has taken the lead in introducing GM cotton (containing Cry1Ac gene, Mon531) varieties to the farming community. These varieties uplifted the livelihood of 1.3 million cotton farmers (as land owners) and >7 million farm laborers. He published more than 10 chapters in foreign books, as well as over 60 publications in Journals including two in *Nature* as co-author. All these efforts were acknowledged by the International and National Agencies by bestowing him with several recognitions/awards including Pride of Performance, ICAC Cotton Researcher of the Year 2014.

Dr. Yusuf Zafar, presently working as Chairman at the Pakistan Agricultural Research Council, has 35 years experience in Life Sciences/Biotechnology. He has been the founding team member of the NIBGE, Faisalabad Pakistan and NIGAB, PARC, Islamabad, Pakistan. He established Agricultural Biotechnology Division in NIBGE and developed several GM crops. He mentored over 15 PhD students and published over 200 research articles, including chapters. Dr. Zafar acted in high-profile positions including Director General, PAEC; Minister Technical in Vienna; Project Management Officer, Technical Co-operation–Asia & Pacific, IAEA, Vienna, etc. His services were recognized by the Government of Pakistan that awarded him with civil award in 2004 and the International Cotton Advisory Committee (Washington USA) that bestowed upon him the best Scientist of the Year award, 2012.

Contents

Preface

The aim of editing this book was to share R&D efforts undertaken to study the properties of natural cotton fiber including color and quality, improving the genetics of naturally colored cotton, genomic insights for mitigating the impact of heat—one of the most devastating components depressing cotton production—genetic and genomics of different traits in cotton, genome editing for combating biotic and abiotic stresses, and the development of transgenic cotton as well as their biosafety issues.

In the first chapter, written by Drs Mehboob-ur-Rahman and Yusuf Zafar, the provoking approaches to sustain cotton yield are described. Till date, cotton varieties were developed for cultivation under high-performance environments to achieve the maximum yield. However, the performance of these cotton varieties is much more vulnerable at resource poor farms. The changing climate would further exaggerate the situation by gradual escalation of temperature and depletion of fresh water resources and make cotton crop difficult to grow. In this chapter, possible solutions, including conventional as well as non-conventional procedures for sustaining cotton production, have been discussed.

In the second chapter, genome editing—a way to tailor gene for silencing or enhancing its expression precisely, has been described. Conventionally, mutations were induced randomly using various physical and chemical mutagens—making it difficult to target the gene of interest. Khan and colleagues described a number of approaches that can target the gene of interest very precisely. Among these, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR), and RNA-guided nucleases (e.g., Cas9) were in common use to target the genome. The CRISPR/Cas has shown a great potential to edit genome of complex species including cotton. For example, lateral root formation was improved using CRISPR/Cas system. Its role for silencing undesirable traits in cotton is yet to be established.

In the second section, impact of the Bijective relationship between single and bundle cotton fiber was narrated by Mahjoub and colleagues. In this study, three different fiber types, chosen from a list of twelve cottons, were selected from a large panel of varieties with substantial variations in the physical properties of fiber. These properties are elongation, single fibers and bundle tenacities, work of rupture, etc. They classified fiber based on length and their linear densities for improving precision and behavior of cotton fibers. This information is extremely important for breeders to know about the complex relationship among various fiber arrangement parameters, which ultimately determine the quality of the fabric.

In the third section, a chapter was compiled for discussing the impact of heat—an emerging menace depression of cotton production worldwide—and various strategies to mitigate this stress. In this regard, Zafar and colleagues described that various physiological and biochemical changes occur in cotton plant upon exposure to extreme temperatures (high as

well as low). In this chapter, various breeding approaches coupled with genomic strategies (marker-assisted as well as transgenic selection) were also described for developing resilient cotton varieties.

The second chapter of this section entitled "Genetic Mapping in Cotton" was written by Bardak and his colleagues. In this chapter, progress made toward understanding the extent of genetic diversity in cotton germplasm and identifying QTLs associated with resistance to various diseases including verticillium wilt, plant architecture, stomatal conductance, yield, and fiber qualities was discussed. Authors have also demonstrated the role of genome-wide association studies (GWAS) coupled with next-generation sequencing (NGS) in identifying DNA markers as well as cloning genes conferring various traits of interest. This information would help in initiating cotton breeding by design.

The third chapter of this section solely focused on updates on "fiber genomic studies." In this chapter, Ayubov and colleagues highlighted the importance of producing high-quality cotton to make the natural fiber more competitive than that of the synthetic fiber. Recent progress on sequencing the whole genome of cotton species together with parallel evolution of bioinformatic tools made it possible to identify closely linked DNA markers as well as genes responsible for fiber quality traits. Earlier, a number of DNA markers (SSRs, EST-SSRs, and SNPs) associated with fiber traits were developed, and few of them were used in marker-assisted selection (MAS). In this chapter, information about the new genomic resources, characterization of cotton genomes, discoveries of many novel genes, regulatory elements including small and micro-RNAs, and novel tools such as gene silencing or gene editing techniques for genome manipulation was provided comprehensively.

In the last section of the book, the impact of transgenic cotton containing Bt genes on non-targets has been reviewed comprehensively by Arshad and colleagues. It has been shown that transgenic crops may disturb the population structure of non-target insect unintentionally. For instance, GM cotton containing Cry genes has been allowed for cultivation to control lepidopterous pests; however, the introduction of GM cotton may support the survival of new insects and may attain the status of pests. The change in species population structure may influence IPM programs, which should be addressed by conducting some planned studies on target and non-target insect diversity. In this chapter, authors have compiled studies of various labs showing the impact of Bt cotton against major target and non-target insect pests in cotton agro-ecosystem. It was concluded that GM cotton is highly specific to the targeted pests and has no negative impact on non-target insect species. The decreased use of insecticides on Bt cotton demonstrated the positive impact on beneficial insect populations and can also support the survival of rare species. It has been shown that the cultivation of Bt cotton is safe and is an environment-friendly approach.

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Section 1

Future Trends in Cotton Research

Chapter 1

Introductory Chapter: Updates on Achieving Sustainable Cotton Production

Mehboob-ur-Rahman and Yusuf Zafar

Additional information is available at the end of the chapter

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1. Introduction

Cotton, a major source of natural fiber and vegetable oil [1], belongs to the genus *Gossypium*. The word cotton derived from "quotn", an Arabic word [2], and *Gossypium* derived from "goz", an Arabic word [3]. The genus *Gossypium* is composed of ~50 species; out of these five are tetraploids (2n = 4x = 52) which evolved about 1–2 million years ago [4–6] through hybridization of two diploid species containing genomes much similar like "A" (A2 (*G. arboreum*) and A1 (*G. herbaceum*)) and "D" (*G. raimondii*). Among these, *G. hirsutum* (AD1) and *G. barbadense* (AD2) are cultivated, while three species including *G. tomentosum* (AD3), *G. mustelinum* (AD4), and *G. darwinii* (AD5) are wild which are endemic to Hawaii, Brazil, and Galapagos Islands. In total, 45 are diploids containing one genome each of the total eight different genomes (A–G and K) [4].

Significant progress toward increasing lint yield and improving lint quality has been made during the last seven decades through using conventional and nonconventional breeding approaches. Transgenic cotton conferring resistance to chewing insect pests as well as to glyphosate not only reduces the cost of production but also increases the yield per unit area in developing countries. Cultivation of transgenic cotton helped IPM programs as well as reduced the toxic impact of pesticide in the environment. Also, the cultivation of *Bt* cotton was found safe for the nontargets. At the moment, cotton is cultivated on >30 million hectares in 80 different countries of the world [7, 8]. World cotton average yield is fluctuating over the last 3 years, and significant reduction (~9% in 2015–2016 than that of 2014–2015) in yield was observed [9]. There are a number of factors including changing climate, resistance development in target insect pests and weeds, increased heat and drought stress, excessive rains and water logging, evolution of new strains of diseases, etc. contributed toward the decline in yield [10, 11]. Another menace is the high demand of inputs for harvesting acceptable yield from *Bt* cotton. Also, the synthetic fiber is competing out the natural cotton fiber owing to its reduced

cost in the international market [3, 10, 11]. Thus, extraordinary efforts are needed to sustain cotton production by 2050.

2. Possibilities toward achieving sustainable cotton production

Bringing sustainability in cotton production is a major challenge for the resource-poor farming communities—dominantly living in developing countries. Other than releasing cotton varieties expressing high yield potential by growing in high input environments—progressive farmers can afford, there is a need to open R&D fronts for developing cotton varieties which can withstand the impact of changing climate and produce sustainable yields in low input or in optimum input environments [11]. Deployment of high-tech genomic tools in breeding is one of the approaches to initiate breeding by design aiming at the development of resilient cotton cultivars [12, 13].

Efforts for achieving maximum yield potential under optimum input environments are not successful largely because of lack of genetic diversity among the genetic resource use to develop new cotton varieties. Narrow genetic base does not only limit the future breeding progress but also makes the crop vulnerable to insect pests and diseases and also to the negative impact of changing climate. For enhancing the genetic diversity of newly developed cultivars, underutilized genetic resources, land races, obsolete varieties, old accessions, etc. are the golden assets for cotton breeders to cultivate genes conferring novel traits [11]. Usually, cotton breeders avoid using the genetic resource from undomesticated germplasm as it hinders the breeding progress because of the linkage drag of some undesirable traits present in the wild germplasm. For having success using conventional breeding approach, selection of diverse parent genotypes, appropriate population size, and accurate testing of newly developed lines in a given ecosystem are the prerequisites to develop cotton varieties with improved genetics [14].

After sequencing of *Arabidopsis* genome, a number of crop species including cultivated cotton species have been sequenced. Also, the progenitor species, i.e., A-genome and D-genome, of the tetraploid-cultivated cotton species have been sequenced. The next question is to understand the extent of genetic diversity residing in genomes, and associating the diversity in gene spellings with phenotypic diversity or important traits (quality, productivity, resistance to biotic and abiotic stresses, etc.) would remain a major challenge [15]. In this regard, massive re-sequencing of thousands of cotton genotypes/accessions of the same species as well as different species would help in elucidating gene function of important traits [16] as well as the footprint of selection [15]. In the present scenario, the function of identical genes can also be deduced by comparing with the similar genes present in *Arabidopsis thaliana*—shared common ancestry with cotton ~83–86 million years ago [15]. Thus, many genes which are analogous to genes present in *Arabidopsis* can be characterized with varying degree of success. However, genes conferring traits typical to cotton will have to characterize by deploying various molecular assays including finding DNA markers associated with the traits using nested-association mapping (NAM) populations, genome-wide association study (GWAS) analysis, etc. or by

using some other forward and reverse genetic approaches—VIGS and CRISPR-Cas system may have a role in the future for assigning functions to different genes [12, 17].

Another important genetic resource in the form of genetic maps construed using several mapping populations developed through interspecific (*G. hirsutum*/*G. barbadense*) and intra-specific (*G. hirsutum*/*G. hirsutum*) crosses is available. Initially, this information may help in developing cotton cultivars by design. Unlike rice, deployment of DNA markers in routine cotton breeding is limited to simple traits; however, selection for complex traits is yet to be realized even in advanced countries. For example, ~1000 QTLs for ~40 different traits have been reported. In total, over 49 genetic maps and about 25,000 genetic markers have been reported in different research articles. This information can be used to breed "designer cotton varieties" [18].

In developing countries, cotton breeders are not using the diagnostic markers linked with simple traits because of lack of resources and trained manpower. It is extremely important to identify DNA markers close enough to the complex traits in spite of the fact that genetic base of the parent genotypes is narrow. In this regard, collaborative efforts among few labs for searching new DNA markers have been initiated by deploying new assays like nested-association mapping and association mapping approaches. Such coordinated efforts can be successful if the goal is common, for example, breeding for resistance to lepidopteron insect pests, drought, and salinity. However, there are few research issues typical to a specific region. For example, cotton leaf curl disease infects cotton in Pakistan and is a potential threat to all other cotton-growing countries where whitefly is prevalent. For addressing this issue, collaborative and coordinated research programs including sharing of cotton germplasm for screening in the hotspot regions would help in tackling such issues. USDA screened >5000 accessions of cotton in hotspot regions of Pakistan, and more than a dozen of asymptomatic cotton genotypes were identified [11, 13, 19]. This information and genetic material are useful for Pakistan and also for the whole cotton-growing community as the threat is spreading to other countries (reported in India and China). Thus, cotton production would be sustained. There are 53,000–63,946 cotton germplasm accessions that are preserved in gene banks of different cotton-growing countries. Sharing these resources is extremely important to characterize the extent of phenotypic and genotypic diversity present in the genus *Gossypium*. A high-throughput phenotyping platform is required to study traits precisely in a large number of genotypes/accessions in the shortest possible time [16]. The use of such automated technologies will expedite the progress toward initiating marker-assisted selection as well as elucidating various genetic circuits of simple and complex traits. Duplicated accessions can be discarded to avoid redundancy. Selected genotypes/accessions reflecting the maximum phenotypic diversity can be explored using genomic tools including SSR-based characterization, genotyping by sequencing, and re-sequencing, together with the application of association mapping analysis including nested-association mapping (NAM) and genome-wide association study (GWAS) methods [20].

Another important genetic resource is the cultivated *G. arboreum* and *G. herbaceum* species. These have been evolved in drought-prone regions and are still under plow in marginal lands of Indo-Pak regions [21, 22]. These two species have relatively greater root biomass than that of

the cultivated *G. hirsutum* species. Thus, these species harbor important genes for high root biomass including deep root system and scavenge water from deep layers of soils. These two species are also resistant to cotton leaf curl disease and other biotic stresses. In spite of the fact that the genomes of *G. arboreum* and *G. herbaceum* have been sequenced, a limited number of genetic maps using diploid interspecific populations have been reported. Identifying QTLs associated with traits typical to these diploid species would be another priority research area for initiating DNA-based selection procedures—previously not possible. The negative linkage drags can be avoided in desirable plants in segregating and/or backcross populations by monitoring the introgression of desirable alleles/genes using the associated DNA markers. Most times, backcross breeding scheme is deployed to recover the genome of recurrent genotype while retaining the desirable alleles of the donor genotype. In the whole scheme, extensive backcrossing followed by identifying plants containing the desirable alleles using DNA markers would be an important breeding procedure for developing improved cotton germplasm.

The use of mutagens for breaking the negatively linked traits has been used to develop new cotton varieties. Earlier radiations (gamma rays) have been extensively used during the early 1960s—induced changes in structure of chromosomes randomly. Usually, soaked seed and/or pollen grains are exposed to physical mutagens. These mutagens can be used to expose F_1/F_2 seed developed through crossing two different species, and the best mutant plants can be identified by surveying with DNA markers originating from the adapted species. This technique was not largely found worth working because of induction of several deleterious mutations and unwanted linkages in the newly developed mutated genotypes.

The use of chemical mutagens is another approach for inducing mutations by exposing the cottonseed with known mutagens; that is, EMS would not only be helpful in assigning functions to different genes but would also be instrumental in adding new alleles through adding or deleting or replacing nucleotides in the genes which would enhance the genetic diversity—a potential buffer to the epidemic of insect pests and diseases.

Hybrid vigor, increased growth over the parent genotypes, has been explored in corn that resulted in multifold increase in production worldwide. Such efforts were also translated in vegetable and arable crops including cotton but could not gain the popularity like corn. For example, in few parts of the world, cultivation of hybrid cotton has shown increase in lint production. The hybrid cotton can be exploited if the hybrid seed surpasses the yield by 30% over the open-pollinated variety (OPV); resultantly, farmers may get rewards of their investments made to procure costly hybrid seed. In this regard, extensive studies are needed to identify the best combiners. Hybrid cotton breeding is also handicapped due to the nonavailability of reliable genetic as well as mechanical means for getting rid of anthers/pollens. Farmers may produce hybrid seed sufficient to meet their own demands (farmers in few provinces of China do this practice) for sowing cotton hybrids instead of OPVs. Thus, farmers can save money meant for buying the hybrid seed. Trainings for the production of hybrid cottonseed may be given to farmers by the public sector organizations [11, 22].

In most developing countries, for example, in Pakistan, the cost of cottonseed is paid based on total weight of the cottonseed irrespective of the lint potential which encourages farmers to

grow varieties producing more cottonseed yield per hectare rather than more lint yield per hectare. A significant fluctuation in lint percentage (35–50%) in the cotton germplasm has been found. Thus, emphasis should be made to breed for >45% lint recovery which would add a couple of million bales toward the total cotton production.

Another breeding objective is to improve the lint quality—always remained a major challenge for breeders as the trait is controlled by multiple thousands of genes. Through conventional breeding, limited success has been achieved; however, further improvement is handicapped due to lack of compatible genetic resources. The success of resolving the complex traits has been demonstrated in model plant species using modern genomic tools. Currently, efforts are underway to clone QTLs involved in conferring these complex traits in cotton. Genome sequence information of the cultivated species also unraveled the genetics of fiber initiation—relatively simpler than that of the fiber elongation traits. Once the key genes involved in defining lint characters are identified and these can be used to engineer the pathways of the diploid cotton species like *G. arboreum* and others, thus fiber production can be sustained in low input environments. Another immediate thought is to initiate projects for "re-sequencing" the representative genotypes of the closely related cotton species using next-generation sequencing approaches which would be a way forward for associating genetic variations with the traits using bioinformatics tools—would add synergy for sustaining cotton production. These studies would also help in identifying new tissue-specific promoters, unlike the constitutive promoters, which may save plant energy.

Protection to insect pests and diseases has been engineered in different crop species including cotton using different novel genes (Cry genes) excised from a soil bacterium. Success of *Bt* cotton in protecting cotton crop from bollworms has been demonstrated since its release in 1996. Now, resistance conferred by the *Cry1Ac* gene has been weakened. Also, pink bollworm infestation on GM-cotton in India has been reported (scientific evidences are lacking). Potential of minor pests for emerging as major pests is another threat to cotton sustainability. For example, before the cultivation of *Bt* cotton in Pakistan, mealy bug and dusky bug have never been problematic to cotton as indirectly controlled by the application of insecticides applied to kill lepidopteron insect pests, but on *Bt* cotton these two insects infested in the recent past. This scenario may also arise in other countries. The situation can be mitigated through educating cotton farmers for monitoring and controlling the new emerging pests by taking measures including the applications of insecticides. Till now, GM-cotton containing few genes (largely of Cry series and genes conferring resistance to glyphosate) has been commercialized [23, 24]. Thus, new genes and/or their transcription factors conferring tolerance to biotic and abiotic stresses from other plant sources including wild species can be characterized followed by the introduction in cotton. These genes would have high chances of acceptance by the end user. Improvement in expression of transgene can be made by designing effective gene cassette with efficient promoters, followed by identification of the best event with high gene expression [17].

Identification of new marker genes (e.g., genes conferring fluorescent proteins) instead of using conventional marker genes (e.g., antibiotic-resistant gene) would be useful for making the use of GM foods more safe. These genes should be tested for an extended time

period on the number of different model organisms for making the conclusions acceptable to the public in effective manner. All these new genes from alien background may be supplemented, or resistance can be delayed by adopting some other strategies including development of short-duration varieties and characters offering defense umbrella (small leaves, pubescence, light green leaves, etc.) for avoiding any significant damage by the insect pests and diseases. Secondly, research on other aspects such as chemical ecology would add synergy in managing eco-friendly insect pests and diseases and thus can enhance yield.

Genome editing through CRISPR-Cas is an emerging tool which can be used to edit the genes to improve or silent their expression. For example, gossypol-free cottonseed can be produced by silencing the genes conferring gossypols in seed. Major advantage of this assay is that the function of gene can be characterized and new cultivars can be evolved without introgressing foreign gene; hence, the technology will be acceptable to countries having skeptical views about the GM technology [11, 12, 15, 17]. Thus, it is summarized that the adoption of high-tech management practices, utilization of untapped genetic resources in breeding, cultivation of cotton varieties with excellent genetics, monitoring of risk and efficacy of transgene in ecosystem, and continued search for new genetic resources would help in sustaining cotton production [11, 12].

3. Conclusions

Cotton breeding, largely based on recombination genetics, has paved the way to develop cotton varieties which were heat tolerant, early maturing, and high yielding with improved fiber traits. Historically, the breeding subject of cotton revolves around the evolution of new cultivars, maintenance of varieties, and their seed production. Presently, phenotypic-based selection procedures have been changed to DNA-based selection systems (marker-assisted selection)—made possible for evolving cotton varieties with brilliant genetics in the shortest possible time. Finding the enormous number of variations has been greatly facilitated by the advent of next-generation sequencing tools, and these variations were assigned functions using advanced bioinformatic tools. All these discoveries and effectiveness of the technologies have made it possible to initiate breeding by design using DNA markers as well as by precise genome editing. Unlike conventional breeding practices, genes (e.g., Cry1Ac, etc.) have been transferred from alien sources for improving resilience to chewing insect pests and herbicides. In the present book, research efforts representing a wide range of research endeavors, being undertaken in different parts of the world, were comprehensively discussed. Both the editors (Drs. Mehboob-ur-Rahman and Yusuf Zafar) ensured the compilation of high-quality research, opinions, and progress made toward enhancing and understanding the cotton genome and its application for developing resilient cotton varieties—a way to sustain cotton production beyond 2050. In the end, editors acknowledge efforts and hard work of the authors in compiling their respective chapters.

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Chapter 2

Targeted Genome Editing for Cotton Improvement

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Muhammad Salman Mubarik and Aftab Ahmad

Additional information is available at the end of the chapter

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Abstract

Conventional tools induce mutations randomly throughout the cotton genome—making breeding difficult and challenging. During the last decade, progress has been made to edit the gene of interest in a very precise manner. Targeted genome engineering with engineered nucleases (ENs) specifically zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided nucleases (e.g., Cas9) has been described as a "game-changing technology" for diverse fields as human genetics and plant biotechnology. In eukaryotic systems, ENs create double-strand breaks (DSBs) at the targeted DNA sequence which are repaired by nonhomologous end joining (NHEJ) or homology-directed recombination (HDR) mechanisms. ENs have been used successfully for targeted mutagenesis, gene knockout, and multisite genome editing (GenEd) in model plants and crop plants such as cotton, rice, and wheat. Recently, cotton genome has also been edited for targeted mutagenesis through CRISPR/Cas for improved lateral root formation. In addition, an efficient and fast method has been developed to evaluate guide RNAs transiently in cotton. The targeted disruption of undesirable genes or metabolic pathway can be achieved to increase quality of cotton. Undesirable metabolites like gossypol in cottonseed can be targeted efficiently using ENs for seed-specific low-gossypol cotton. Moreover, ENs are also helpful in gene stacking for herbicide resistance, insect resistance, and abiotic stress tolerance.

Keywords: TALENs, CRISPR/Cas, DSBs, NHEJ, HDR, cotton

1. Introduction

Cotton is an important source of natural fiber and has been playing a major role in economy and social structure of several countries. In addition, cotton serves as cash crop for more than

20 million farmers in Asia and Africa. Despite the availability of synthetic alternatives, cotton remains an important source of fiber because of the advantages related to cost of production and unique features offered by cotton lint. Consumption of cotton products in the world is increasing day by day with a lot of paces, but world cotton production is stagnant because of biotic and abiotic stresses. To meet the demands of the masses, production of cotton needs to be very high with good quality. Cotton is also affected by diseases, causing significant losses to industry. The most damaging diseases are Texas root rot, bacterial blight, blue disease, cotton leaf curl disease (CLCuD), and some strains of *Verticillium* and *Fusarium* wilt. Abiotic factors (heat, drought, salinity, and waterlogging) affect cotton yield, especially during early stages of plant development. Along with conventional breeding and genetic engineering, other novel techniques such as GenEd could be helpful for resistance development in cotton against biotic and abiotic stresses. GenEd tools have also been used for growth, quality, and yield enhancement in other crop plants. So, translation of this marvelous technology for improvement of fiber, quality, and yield of cotton would definitely have long-lasting benefits. In this chapter, we provide a picture of the use of GenEd tools for genetic improvement of cotton and other crop plants.

2. GenEd tools for targeted genome modification

Mutagenesis at target sites was a long-standing goal in the field of genome engineering and biotechnology. Along with chemical mutagens, transposons, recombinases, and TILLING technologies have been used historically to mutate certain genes for functional genomics and reverse genetic studies. The last decade has observed a revolution in the field of targeted genome modifications. GenEd has been found successful with equal efficiency in both plants and animals. Targeted genome modifications have modernized the field of genome engineering and biotechnology by GenEd from unicellular to multicellular and from prokaryotic to eukaryotic organisms. A diversity of organisms from bacteria to humans such as *Arabidopsis thaliana* [1], tobacco [2], rice [3], yeast (*Saccharomyces cerevisiae*) [4], fungi [5], zebrafish [6], rats [7], sheep [8], *Caenorhabditis elegans* [9], human cell lines [10], *Drosophila* [11], viruses [12–14], bacteria [15], mouse [16], insects [17], cattle [18], goat [19], pigs [20], tomato [21], grapes [22], potato [23], soybean [24], maize [25], wheat [26], and cotton [27, 28] have been targeted successfully with engineered proteins and nucleases.

GenEd tools like zinc-finger nucleases, transcription activator-like effectors, and CRISPR/Cas have been used massively for targeted genome modification. These GenEd reagents have the ability to search and bind specific DNA sequence and, hence, can be programmed to target any DNA sequence of choice. All of the ENs mentioned above have a catalytic ability to create double-strand breaks (DSBs) at the target DNA sequence. Zinc fingers and TALEs are fused with FokI nuclease domain to induce DSBs on dimerization, while CRISPR/Cas9 has its own catalytic activity with two nuclease domains: RuVC and HNH. DSBs at a predefined DNA sequence can be utilized efficiently for targeted genome modifications. DSBs in the DNA are repaired through cell endogenous repair systems: nonhomologous end joining (NHEJ) and homology-directed recombination. NHEJ is an error-prone repair mechanism in which DSBs are repaired with some insertions and/or deletions (Indels). On the provision of a homologous

DNA template or donor DNA, the DSBs are repaired without errors in HDR fashion. HDR is an efficient pathway to make targeted insertions and/or gene corrections.

Reprograming and redesigning of artificial DNA-binding proteins and ENs have made GenEd quite an easy job. Most of the softwares are freely available online for the designing and cloning of ENs. Apart from ZFNs, TALENs, and CRISPR, other ENs such as homing endonucleases or meganucleases (DADGILAGLI) have also been used for targeted GenEd [29], but their applicability is very low compared to the above-mentioned nucleases.

2.1. Zinc-finger nucleases

The first targeted induction of DSBs was achieved using the natural meganuclease I-SceI, which has an 18-bp recognition site [30]. Experiments performed in tobacco using I-SceI to introduce chromosome breaks at integrated, defective reporter genes which, upon correction by homologous recombination, confer a selectable phenotype [30, 31]. Zinc fingers were fused with FokI nuclease to create artificial endonuclease for targeting predetermined DNA sites [32]. Zinc-finger nuclease-assisted gene targeting was first implemented in animal systems [33]. In the late 1990, ZFNs were designed and used for the first time to target genes of *Drosophila melanogaster* [34]. In case of ZFs, three DNA bases are targeted with one monomer. ZF monomers have been deciphered, and a table was built with possible combinations of three DNA bases to design ZFs against a DNA sequence (**Figure 1a**). Two efficient ZFN assembly platforms are available for successful designing and cloning of ZFNs: oligomerized pool engineering (OPEN) [35] and context dependent assembly (CoDA) [36]. Previously, modular assembly method was used to assemble multi-finger ZFN arrays, but the efficiency was reported low owing to inefficiency for context-dependent activity.

Using two ZFN monomers results in DSB formation by a functional nuclease dimer, as initially shown for FokI endonuclease coupled to three ZFs636 recognizing 9-bp-binding sites [32, 37]. Induction of ZFN expression in *Arabidopsis* by heat shock during seedling development resulted in mutations at the ZFN recognition sequence. In 10% of induced individuals, mutants were present in the subsequent generation, thus demonstrating efficient transmission of the ZFN-induced mutations [38]. Homologous recombination was measured by restoring function to a defective GUS:NPTII reporter gene, integrated at various chromosomal sites in ten different transgenic tobacco lines [39]. ZFN-mediated gene targeting at endogenous plant genes of tobacco acetolactate synthase genes (ALS SuRA and SuRB) was observed with high frequency exceeding 2% of transformed cells. Targeting of SuR loci resulted in resistance to imidazolinone and sulfonylurea herbicides with allelic mutations [40].

Co-expression of ZFNs with heterologous donor molecule led to precise targeted addition of an herbicide tolerance gene at the intended locus in maize. Mutant maize plants also transmitted genetic changes to further generation [41]. HDR-based gene replacement has been achieved successfully by replacing a 7-kb fragment flanked by two ZFN cutting sites with a 4-kb donor cassette, which integrates genes of kanamycin resistance and red fluorescent protein (RFP) [42]. In the last decade, artificial zinc-finger proteins (AZPs) have been used against begomoviruses (beet severe curly top virus (BSCTV) and tomato yellow leaf curl

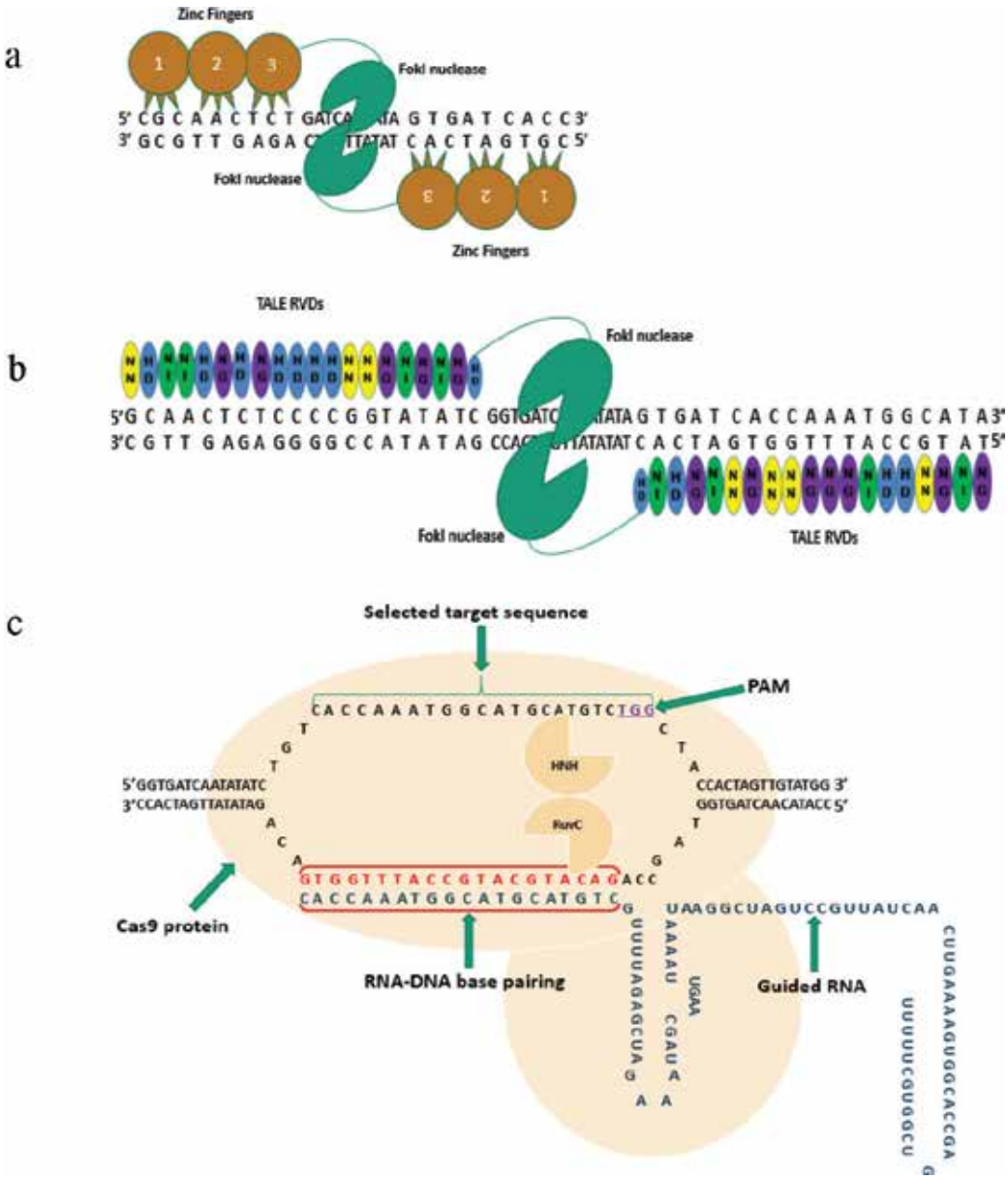

Figure 1. Genome editing tools: (a) a pair of ZFNs with zinc-finger monomers and a pair of FokI nuclease to cut a dsDNA, (b) a pair of TALENs with TALE-binding repeats and FokI nuclease domains, and (c) CRISPR/Cas9 targeting dsDNA along with sgRNA and nuclease domains to create DSBs.

virus (TYLCV), respectively) [43, 44]. This strategy can be used for suppression of begomoviruses infecting cotton plants [45]. Moreover, ZFNs and AZPs can be useful for gene insertion, deletion, replacement, and functional genomics studies in cotton. Selected reports of ZFN-mediated genome modification are given in **Table 1**.

Sr. #	Plant species	Gene	Gene modification	Reference
1	Maize	*IPK1*	NHEJ	[35]
2	Tobacco	*SuRA*, *SuRB*	NHEJ	[40]
3	*Arabidopsis*	*ADH1*, *TT4*	NHEJ	[41]
4	*Petunia*	*mGUS*	NHEJ	[132]
5	Soybean	DCL	NHEJ	[24]
6	Tobacco	*Kan*, *RFP*	HDR	[42]

Table 1. ZFN-mediated genome modifications in plants.

2.2. TALEs and TALENs

TALE proteins are bacterial proteins (plant pathogens: genus *Xanthomonas*) and produced to bind DNA in the infected plant to hijack the expression system in a way that attenuate the disease process. Natural TALEs have a binding domain and an effector domain which binds DNA sequence and alter expression system of host, respectively. The binding domain consists of variable number of amino acid repeats in which each repeat contains

Organism	Gene	Editing	Reference
Arabidopsis	*ADH1*	NHEJ	[1]
Tobacco	*EBE* of *Hax3*	NHEJ	[2]
Rice	*EBE* (*AvrXa7* and *PthXo3*)	NHEJ	[3]
Potato	*Vlnv*	NHEJ	[23]
Wheat	*MLO*	NHEJ	[26]
N. benthamiana	*FucT*, *XylT*	NHEJ	[54]
Rice	*OsSD1*, *OsBADH2*	NHEJ	[139]
Brachypodium	*BdABA1*, *BdSPL*	NHEJ	[139]
Tobacco	*SuRA*, *SuRB*	NHEJ, HDR	[140]
Barley	*PAPhy_A*	NHEJ	[141]
Brassica oleracea	*FRIGIDA*	NHEJ	[142]
Soybean	*FAD2–1A*, *FAD2–1B*	NHEJ	[109]
Barley	*PAPHY-A*	NHEJ	[143]
Rice	*OsMST8*, *OsMST7*, *OsEPSPS*	NHEJ	[144]
Maize	*Glossy2 locus*	NHEJ	[145]
Arabidopsis	*CLV3*	NHEJ	[146]

Table 2. Genome editing in plants through TALEN technology.

33–35 amino acids and recognizes a DNA base pair. The DNA recognition is specifically modulated by two hypervariable amino acid residues (also called as repeat variable diresidues (RVDs)) at positions 12 and 13 in each repeat. Therefore, TALE repeats can be engineered by varying the RVDs to create a TALE protein that can bind a specific sequence in the genome (**Figure 1b**).

In case of TALEs and TALENs, the designing and assembly can be done with more ease and comfort. Owing to the single base-pair specificity of the TALE RVDs, modular assembly has been used frequently. Golden gate assembly of Cermak et al. has advantage of being fast, simple, and cost-effective [1]. Many free online softwares are available to design TALEs and TALENs [45]. The assembly of TALENs has also been offered on commercial basis by different companies, and many kits are available to construct TALENs against target sequence [45]. These TALE domains can be linked with a designed effector domain (nuclease like Fok1, repressor like KRAB, or activator like VP64) to create a chimeric protein capable of targeted genome manipulation. Successful genome modifications have been achieved using TALENs in different plant species (**Table 2**).

2.3. CRISPR/Cas RNA-guided system

CRISPR/Cas is an RNA-guided endonuclease (RGEN) system. RGENs are the easiest and simplest to design and clone. Cas9-gRNA is based on simple Watson and Crick base pairing of RNA-DNA, and 20-bp guide RNA is designed to target a DNA sequence of interest (**Figure 1c**). The efficiency of RNA-guided Cas9 system is remarkable to rewrite genomic sequence for genetic improvement of crops against different threats of multiple origins. Due to the ease of designing, simplicity in cloning, and cost-effectiveness, CRISPR/Cas is the most widely used EN.

CRISPR/Cas has emerged as a new tool for targeting DNA using single-guide RNA (sgRNA), enabling genetic editing of any region in the genome [46, 47]. This single RNA-single protein CRISPR system is derived from a natural microbial adaptive immune system that uses RNA-guided nuclease to recognize and cleave foreign DNA elements. This system consists of two components, a chimeric sgRNA and a CRISPR-associated protein (Cas9), which specifically unwinds and cleaves the target DNA, with the cleavage site dictated solely by complementarity to the sgRNA [48]. The only restriction in this system to target a DNA sequence is the presence of protospacer adjacent motif (PAM) region. CRISPR system has been proven to be incredibly valuable for site-specific genome engineering. Recently, in bacterial and human cells, nuclease's deactivated version of Cas9 protein called as dCas9 was created for programmable RNA-dependent DNA-binding protein [49]. Targeting nuclease-inactive Cas9 protein (dCas9) to coding region of a gene can block the binding and elongation of RNA polymerase, leading to dramatic suppression of transcription. Moreover, it has also been reported that dCas9 can also be modulated to recruit different protein effectors (activators or repressors) to DNA in a highly specific manner [50] to activate (CRISPRa) or suppress (CRISPRi) a gene. More recently, fusing dCas9 with Krüppel-associated box (KRAB) repressor domain resulted in an efficient transcriptional interference [50, 51]. In addition, CRISPRi was also used for multiplexed control of endogenous genes [52] and stable repression of genes with silencing efficiency typically achieved by RNAi while minimally impacting transcription of nontargeted genes. CRISPR/Cas9 has the efficiency to target the green fluorescent protein (GFP) gene within the genome of transgenic

Plant species	Targeted gene	Modification	References
Arabidopsis	*PDS3, FLS2, RACK1b, RACK1c*	NHEJ	[66]
Barley, cabbage	*HvPM19, BoIC.GA4.a*	NHEJ	[147]
Camelina	*FAD2*	NHEJ	[148, 149]
C. reinhardtii	*CpFTSY, ZEP*	NHEJ	[150]
Cotton	*GFP* (transgene), *CLA1, VP*	NHEJ	[28, 53]
Dandelion	*1-FFT*	NHEJ	[151]
Flax	*EPSPS, BFP (transgene)*	NHEJ, HDR	[152]
Grape	*IdnDH*	NHEJ	[22]
Lettuce, *N. attenuata*	*BIN2, AOC*	NHEJ	[128]
Liverwort	*ARF1*	NHEJ	[153]
Lotus japonicus	*SYMRK, LjLb1, LjLb2, LjLb3*	NHEJ	[154]
Maize	*IPK*	NHEJ	[25]

Table 3. Genome editing in plants through CRISPR/Cas9.

cotton line with single copy of GFP gene incorporated previously [53]. Multiplexing ability of CRISPR/Cas system has given a distinction to this system. Multiplexed, targeted gene editing has been achieved in *Nicotiana benthamiana* for glycol engineering and monoclonal antibody production [54]. CRISPR/Cas system has been used efficiently for GenEd in plants (**Table 3**).

Specific DNA-binding proteins such as zinc fingers, TALEs, and dCas9 can be fused with different effector domains like activators, repressors, and epigenome modifiers to modulate gene expression (**Figure 2**). DSB created by ENs/RGEN can be used for different purposes (**Figure 3**). Controlled and tuneable expression of genes can be tremendously used for genetic improvement of plants. Modification of epigenetic marks can be further saved from regulation

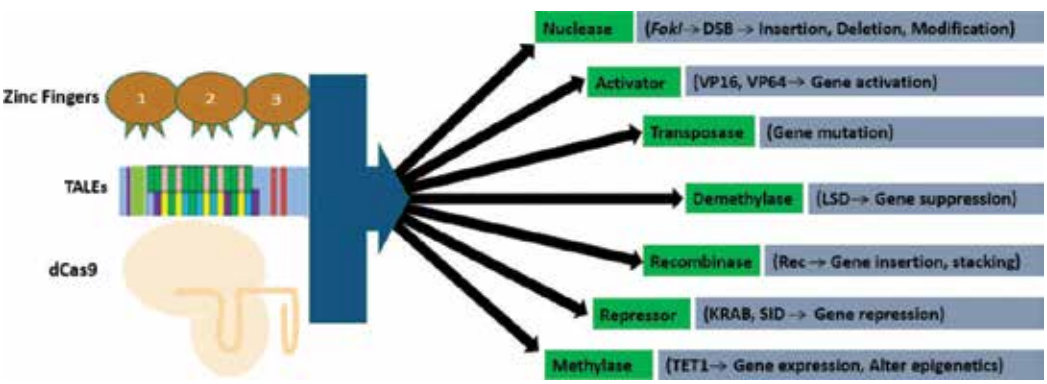

Figure 2. Functional domain engineering of zinc fingers, TALEs and dCAs9, for different purposes. ZFs/TALE/dCas9 proteins can be engineered and fused with different effector/functional domains for targeted genome modifications. In this figure, different functional domains have been shown which can be fused with ZFs/TALE/dCas9 for creation of DSBs, gene insertion, gene activation, gene mutation, gene repression, gene stacking, and epigenetic modifications.

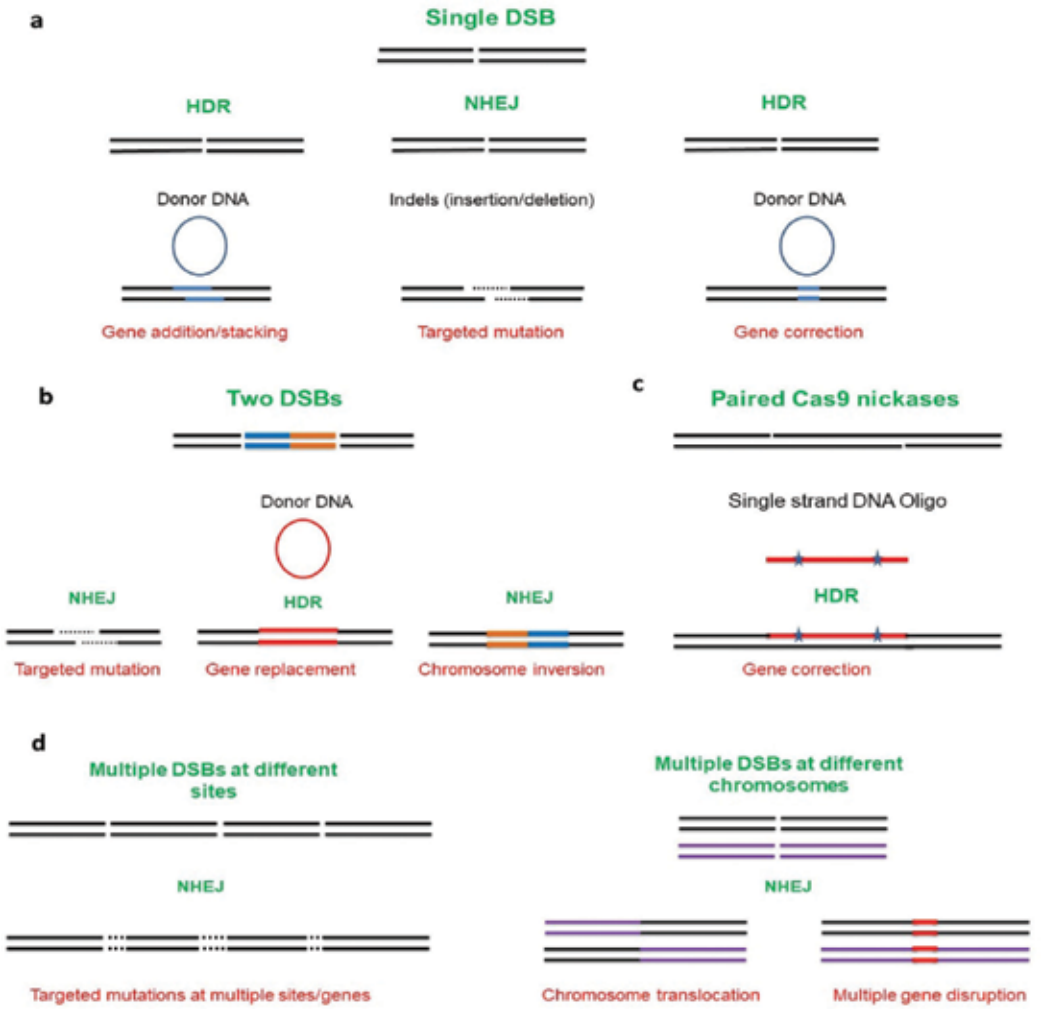

Figure 3. Targeted genome modifications through inducing DSBs. (a) Induction of DSB at the target site using one pair of ENs or one RGEN. DSB is further used to targeted mutations through NHEJ or gene insertion/correction by providing donor DNA. (b) Creation of DSBs using two ENs for targeted mutation, gene replacement/deletion, and chromosome inversion. (c) The use of paired nickases for gene correction and decreasing off-targeting. (d) Multiplexed Cas9 can produce DSBs at different sites for targeted mutagenesis, or induction of DSBs at two different chromosomes may lead to multiple gene disruption or chromosome translocation.

as GMOs. ZFNs, TALEs, and TALENs and Cas9, dCas9, and multiplexed Cas9 can be used efficiently for genetic improvement of cotton through gene deletion, insertion, replacement, correction, and modulation of expression.

3. Use of GenEd tools against abiotic stresses in cotton

Abiotic stress is a multigenic and complex trait. A substantial interaction between several components of signaling, regulatory, and metabolic pathways leads to response/adaptation

to abiotic stress [55–57]. In response to abiotic stress, sometimes, plants may undergo whole-genome duplication events, and functional redundancy in multigene families may also be observed. Single-gene knockout often produces undesirable results/phenotypes making difficult to unravel the exact function. A comprehensive understanding of molecular basis of abiotic stresses (including drought, salinity, and heat) and their tolerance mechanisms have been one of the major goals of plant researchers to engineer stress tolerance in plants.

A VIGS-mediated gene silencing of sucrose non-fermenting-1-related protein kinase 2 (GhSnRK2) mitigated drought tolerance in cotton plants, indicating that GhSnRPK2 positively conditions drought stress and low-temperature tolerance [58]. Moreover, RNAi of cotton PHYA1 genes improved drought, salt, and heat tolerance in transgenic plants, due to increased photosynthesis and better developed root systems [59]. This kind of genes can also be targeted for deletion with pair of ENs or RGENs. Moreover, ZFs, TALEs, and dCas9 can be used for suppression of such genes at the transcriptional level.

To increase the tolerance in cotton against drought stress, transcription factors are excellent candidates for the plant scientists. Various transcription factors (such as MYB, WRKY, ERF, NAC, bZIP) are involved in normal development as well as in drought stress response. These transcription factors have been cloned and proven useful for stress tolerance in cotton and/or in other plants. The genetic engineering of transcription factor genes could activate drought tolerance pathways and enhance drought tolerance in cotton. Recently, a bZIP transcription factor gene, GhABF2, has been reported in the drought and salt tolerance in *Arabidopsis* and cotton. The transcriptomic analysis revealed that GhABF2 regulates genes related to ABA. Overexpressing GhABF2 in cotton increased SOD and CAT activities as compared to wild-type plants. Moreover, overexpressed plants showed better results in the field, and meanwhile its yield was recorded higher than wild-type plants [60]. Stacking of these gene/transcription factors in best-growing cotton varieties with strong promoters could produce more resistant varieties. In another case, overexpressing GbMYB5 positively involved in response to drought stress in cotton and tobacco by reduced water loss from stomata and showed hypersensitivity to ABA [61].

Mitogen-activated protein kinases (MAPKs) are important signaling molecules that respond to drought stress. In a study, SlMAPK3 was induced by drought stress, and CRISPR/Cas9 system was utilized to generate SlMAPK3 mutants [62]. Field tests of transgenic maize plants with reduced ethylene biosynthesis by silencing 1-aminocyclopropane-1-carboxylic acid synthase 6 significantly improved grain yield under drought stress conditions [63]. Similarly, decreasing the sensitivity of maize to ethylene also resulted in higher yield [64]. Overexpression of ARGOS genes and negative regulators of the ethylene response enhances drought tolerance in transgenic maize plants [64, 65].

Due to its simple design and efficient cloning of single or multiple gRNAs, CRISPR/Cas9 system using multiplex genome editing represents a promising and very powerful tool to specifically modulate the expression and activity of genes involved in abiotic stress responses. Multiplexing through CRISPR/Cas9 has been used successfully in model and crop plants [19, 66, 67]. Multiplex genome editing may also be useful for studying functions of gene families as well as an interaction between multiple genes. Multiple genes involved in stress regulatory network, signal transduction, and metabolite production may be simultaneously targeted

via CRISPR/Cas9 technologies for engineering stress tolerance in crop plants. An additional strategy could be pyramiding/stacking of multiple stress regulatory genes through HDR-mediated gene targeting.

4. Use of GenEd tools against biotic stresses in cotton

Conventional methods have been used for integrated pest management (IPM). Physical, chemical, and biological methods have been used for pest and disease management since domestication of crops. For insect resistance, the most widely used technology is *Bacillus thuringiensis* (Bt) technology. Through expression of Bt genes, *Cry* toxin, many insect-resistant crops have been developed [68]. Bt crops helped in decreasing insect attack and the use of pesticides and, hence, had done a good job for decreasing pollution as well. But unfortunately, resistance against Bt has been observed in certain parts of the world like resistance in pink bollworm in India. Apart from Bt technology, RNAi technology has also been used for insect resistance in crop plants. The first report of RNAi technology for cotton bollworm resistance was developed [69] by expression of dsDNA of insect-derived cytochrome P450 monooxygenase gene (dsCYP6AE14). Stacking of dsCYP6AE14 and plant cysteine proteases, such as GhCP1 from cotton (*Gossypium hirsutum*) and AtCP2 from *Arabidopsis*, can increase insect resistance in plants against cotton bollworms. In addition, stacking of new genes with old transgenic cotton varieties will further produce durable resistance against insects. Bt alternate transgenic approaches have also been used at the laboratory scale to develop new strategies of insect resistance in plants.

To counter the insect resistance against Bt crops, alternate strategies include expression of other toxins [70], engineering with proteases [71], proteinase inhibitors [72], receptor proteins [73, 74], and double-stranded RNA [75]. Among all these, dsRNA has been proposed as a method of choice and next-generation insecticide [75]. Moreover, expression of small dsRNA of CYP450 genes in transgenic plants to target vital bollworm functions has been reported as alternative to Bt applications [69]. Most recently, CRISPR/Cas9 was used to knock down a male-determining factor gene, *Nix*, in *Aedes aegypti* mosquitoes, leading to partial sex-change phenotypes [76]. The demonstration of using CRISPR/Cas for inhibition of mosquito-borne disease suggests that GenEd tools can also be translated for inhibition of other insect-borne diseases like whitefly that acts as vector for CLCV transmission to cause CLCuD.

Viral diseases are generally controlled by eliminating the vector population which transmits them. Scientists have been using conventional breeding [77, 78], pathogen-derived resistance [79–81], and nonpathogen-derived resistance [82, 83] to control the diseases. Most efforts were focused on silencing gene(s) of helper virus, but genes on satellite molecules were ignored. Such efforts proved effective but for a short period of time, and then virus relapsed because of multiple infections, synergistic effects, and evolution. A variety of multiplex genome engineering models in plants and animals are available either with expressing multiple gRNAs under single RNA Pol-III promoter [84, 85] or under different promoters at the same time [86, 87]. The CRISPR/Cas9 system has been successfully used

for controlling BeYDV [88], BSCTV [13], and TYLCV [12] with very few off-target activities, and these successful reports highlight the enormous potential of CRISPR/Cas system against geminiviruses. Due to inexpensive, simple, and rapid mechanism for triggering site-specific genome modifications, the programmable Cas9-gRNA system is potentially transforming next-generation genome-scale studies. The efficiency of RGEN system is remarkably high for crop improvement against potential threats of multiple origins (viral and bacterial diseases) especially CLCuD.

The strategy of targeting rep gene or rep protein-binding sites to occupy or disrupt the binding sites could be very fascinating using TALE and TALEN approach with high specificity. Recently, it has been demonstrated [89] that artificial TALE proteins could be a platform for broad-spectrum resistance against begomoviruses. Targeting viral DNA or host factors associated with pathogenesis of viral disease for disruption could be the possible strategies for virus suppression and disease resistance. There is a great possibility and progress in the idea of using TALEN and TALE repressors for antiviral gene therapy as well, to suppress potent viruses that cause global mortality and morbidity like HIV [90]. So far, different regions of viral genomes have been targeted to inhibit replication and to suppress viruses. As a result, decrease in titer of the virus by using ENs has been achieved by many researchers [13, 91].

5. GenEd tools for epigenetic modifications in cotton

DNA methylation is generally defined as an epigenetic mark of transcriptional gene silencing. Epigenetic regulation is although mysterious but can be modulated for a desirable change in the genome. Gene regulation without any change in DNA remained a challenge for years, but now factors have been deciphered which are responsible for epigenetic suppression or activation of genes. So, it has become possible with the help of engineered proteins to modulate gene expression of a gene epigenetically as well. So far, ZFs, TALEs, and CRISPR/Cas were dominantly used for this purpose [92–95], but recently TALEs and dCas9 have become available for this purpose. These proteins fused with different effector domains like 10–11 translocation methylcytosine dioxygenase 1 (TET1) [96], lysine-specific demethylase 1A (LSD1) [97], and methyltransferase which have been used as potential epigenome editors. ZFs fused with TET1 (ZF-TET1) were successfully used for demethylation purpose [96]. In addition, TET1 was used in demethylation of cytocine at CpG sites, and LSD1 has been used for demethylation of H3K4me1/2 and deacetylation of H3K27.

DNA methylation is a conserved epigenetic mark important for genome integrity, development, and environmental responses in plants and mammals. Active DNA demethylation in plants is initiated by a family of 5-mC DNA glycosylases/lyases (i.e., DNA demethylases). Repeat regions, promoters, enhancers, and gene body are the main sites for DNA methylation in the genome. Epigenetic regulation also contributes in splicing. Recent reports suggested a role of active DNA demethylation in fruit ripening in tomato [98]. It was revealed that DNA demethylation is required for tomato fruit ripening through both activation of induced genes and inhibition of ripening-repressed genes. DNA methylation controls many aspects of plant

growth and development. TALE-LSD1 was used to modify methylation pattern of different sites, confirmed through chromatin immunoprecipitation [99]. Gao et al. have confirmed that for epigenome modifications, TALEs are more effective than dCas9 in mammalian cells. Moreover, they have evaluated TALE and dCas9, for gene activation and repression purpose, and highlight the use of designed transcription factors for epigenome modifications.

Epigenetic modifications of chromatin at the DNA or histone level are considered to be one of the major forces that influence gene expression [100, 101]. Genome-wide changes in methylation patterns have been linked with physiological and developmental responses. Genetic imprinting in *Arabidopsis* endosperm and embryo was also driven by extensive demethylation of whole genome coupled with hypermethylation of non-CG residues especially CHH sites on transposable elements [102, 103]. In plants, genes, transposons, and repetitive sequences were found to be methylated in different densities at various developmental stages, which suggested that the transcription of certain genes is controlled epigenetically [104, 105]. Indeed, promoter DNA hypermethylation was related to target gene repression in undifferentiated *Arabidopsis* cells [106]. Jin et al. [107] reported that annual pattern of cytosine methylation drives fiber growth in cotton and moreover also studied the degree of CHH DNA methylation in the promoter regions of the growth-regulating genes SUR4, KCS13, and ERF6 on yearly basis.

However, potential application of TALEs for targeting DNA or histone for epigenome editing has been demonstrated, but more research is needed for development and validation of epigenetically modified crops/organism (EMO). About 500 genes have been identified that are epigenetically modified between wild cotton varieties and domesticated cotton, some of which are known to relate to agronomic and domestication traits. By selectively turning gene expression on and off, breeders could create new varieties of cotton without altering the genes.

6. Use of GenEd tools for growth, yield, fiber, and seed quality enhancement

Accelerated breeding of plant species has the potential to help challenge environmental and biochemical cues to support global crop security. Lengthy breeding cycles are one of major limitations in the rapid genetic improvement and commercialization of woody plant species. In recent years, limitation of T-DNA segregation after site-specific genome editing has gained prominence with the widespread use of CRISPR/Cas technology in genetic engineering. CRISPR/Cas platform will help to strengthen molecular breeding and development of resistance against biotic and abiotic stresses as well as yield and quality improvement in cotton [108].

Jiang et al. [149] used CRISPR/Cas9 to target the FAD2 gene in *Arabidopsis thaliana* and in the closely related emerging oilseed plant, *Camelina sativa*, with the goal of improving seed oil composition. *C. sativa* is allohexaploid, while cotton is allotetraploid and, so, can be targeted with ENs to produce quality seeds. For quality improvement of soybean, TALENs were used to mutate two fatty acid desaturase genes FAD2-1A and FAD2-1B [109]. The mutations also improved shelf

life and oxidative stability along with decrease in polyunsaturated fats. RNAi-based silencing of two key fatty acid desaturase genes, GhSAD-1 and GhFAD2-1, in cottonseeds significantly increased stearic and oleic acid contents in transgenic lines. In addition, palmitic acid contents were significantly low in both high-stearic and high-oleic transgenic cotton lines. These results provide an opportunity for nutritional improvement of cottonseed oil through genetic engineering [110]. Engineering of cotton in same manner through CRISPR/Cas9 or TALENs will improve cottonseeds valuable for farmers and oilseed industry.

Cottonseeds contain high-quality protein and oil so it is also an important source of nutrient-rich food crop and edible oil. For every kilogram of fiber collected, about 1.65 kg of seeds are produced. Therefore, cotton can potentially provide the protein requirements of half a billion people if it could be used directly as food. However, cottonseeds are toxic for humans and other monogastric animals because of the presence of gossypol in the seed glands. Gossypol is a toxic terpenoid compound that causes heart and liver damage in human beings. Gossypol-free cottonseed may enhance the overall value of cottonseed and may generate a new market for cottonseed. Therefore, gossypol-free cottonseeds could provide protein requirement to poultry, aquaculture, and millions of humans worldwide. Gossypol is not only mainly localized in cottonseeds but also presents in other parts of cotton plant. In leaves and reproductive tissues of plant, gossypol and other related terpenoids play a protective role against insects, provoking infertility in insects. RNAi has been used successfully to reduce gossypol contents in cottonseeds by silencing (+)-δ-cadinene synthase which catalyzes the very first reaction involving the cyclization of farnesyl diphosphate to (+)-δ-cadinene. However, RNAi has several disadvantages like off-targets and reproducibility [111]. Thus, the promise of cottonseed to ensure food security and protein requirement of the developing countries like Pakistan remained unfulfilled. Recently, Ma et al. [112] have mapped a gene (*GoPGF*), acting as a positive regulator of formation of pigment glandular trichomes, storage organs of gossypol. Tissue-specific silencing of this gene will result in gossypol-free seeds while maintaining the level of secondary metabolites in the other parts of the plant [106]. Targeting dCas9 to regulatory region of a gene can block the binding and elongation of RNA polymerase, leading to dramatic suppression of transcription. Moreover, it has also been reported that dCas9 can also be modulated to recruit different protein effectors (activators or repressors) to DNA in a highly specific manner [50] to activate (CRISPRa) or suppress (CRISPRi) a gene. More recently, fusing TALEs and dCas9 with KRAB repressor domain resulted in an efficient transcriptional interference [51, 113, 114]. In addition, CRISPRi was also used for multiplexed control of endogenous genes [114] and stable repression of genes with silencing efficiency typically achieved by RNAi while minimally impacting transcription of nontargeted genes.

Flowering is a very critical developmental stage in cotton. All of the production depends on flowering. From emergence to drying up or falling off, it takes just 5–7 days. Flowering depends largely on temperature, availability of water, and other environmental conditions. Growth and development stages in cotton, from planting to emergence, from emergence to square, from square to flowering, and from flowering to boll development, are water sensitive. SELF-PRUNING 5G (SP5G) is a repressor of flowering in tomato and drives loss of day length sensitivity in flowering. CRISPR/Cas9-based mutation in SP5G resulted in

compact growth of tomatoes with rapid flowering. Moreover, mutation also caused a quick burst of flowering that resulted in early yield. Early and uniform flowering in cotton can be used for ease in mechanized picking as well. Identification of FLOWERING LOCUS T (FT) [115] gained prominence for its use in advanced breeding initiatives. FT is a small globular protein that interacts with FT-INTERACTING PROTEIN 1 and moves to sieve elements. From sieve elements FT is transported to shoot apical meristem and interact with bZIP transcription factor FD and phospholipid phosphatidylcholine [116] for its nuclear localization. Finally, FT activates LEAFY (LFY), APETALA1 (AP1), and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) to start flowering development [117–119]. Overexpression of FT has been used in many plant species to induce advanced flowering [120, 121], thus enabling a more rapid and refined approach to breeding. CRISPR/Cas has also been used successfully to target dihydroflavonol-4-reductase-B (DFR-B), encoding an anthocyanin biosynthesis enzyme that is responsible for the color of the plant's stems, leaves, and flowers [122]. Moreover, CRISPR/Cas9 system was employed to specifically induce targeted mutagenesis of GmFT2a, an integrator in the photoperiod flowering pathway in soybean [123].

Li et al. [108] proposed applications of CRISPR/Cas system for improvement in cotton growth and development, seed quality, and flowering timing and control. They examined targeted mutagenesis in allotetraploid genome of cotton, and no off-target mutations have been observed by sequencing two putative off-target sites, which have three and one mismatched nucleotides with GhMYB25-like sgRNA1 and GhMYB25-like sgRNA2, respectively.

Proper development of plant roots is critical for primary physiological functions, including water and nutrient absorption and uptake, physical support, and carbohydrate storage. Crop roots are the main organs that primarily sense and respond to the biotic as well as abiotic stresses. Previous studies on crop root development have proven that increased lateral root formation (LRF) has a positive effect on whole plant development as well as crop yield. Functions of cotton root system are also strongly influenced by lateral roots. A high number of lateral roots would increase the total root surface area of the plant that may potentially improve the overall growth, fiber length, yield, and stress tolerance against severe conditions. Therefore, engineering cotton plants for the increased number of lateral roots will not only improve the yield and fiber contents but will also make cotton crop suitable for salt, drought-affected, and low-fertility soils. Recent studies demonstrated that arginine (ARG) is the precursor of nitric oxide (NO) in roots catalyzed by nitric oxide synthase (NOS) [124], and NO plays a key role in the lateral root formation. In *Arabidopsis* reduced activity of arginase may increase NO contents in roots and therefore improved the lateral roots in transgenic plants. Given that there are two, highly similar, orthologous, cotton arginase genes (GhARG), Gh_A05G2143 and Gh_D05G2397, in the A and D chromosomes that were mutated with CRISPR/Cas9 in upland cotton R18, a transgenic acceptor variety bred from the Coker 312 cotton, which is, globally, a main transgenic acceptor germ line [62]. CRISPR/Cas system was efficient in producing targeted mutations in the selected genes which improved lateral root system under both high and low nitric conditions ensuing adaptation of cotton on a variety of soils. Improved LRF will enhance plant growth and development as well.

7. Use of GenEd tools for gene stacking

Genome engineering with the help of recombinases is no longer a new approach. Site-specific recombinase technology is used to delete, insert, or invert a specific sequence at a target site. A transgenic organism with Cre recombinase expressed by a tissue-specific promoter can be crossed to excise the gene present between two loxP sites. Targeted excision deletes the function of genes within specific tissues. Deletion of genes by site-specific recombinase technology is a particularly advantageous method of gene excision [125].

Site-specific recombinases are remarkable tools for insertion of multiple genes on single locus or deletion of unwanted sequence from the genome. With discovery of ENs, sequence-specific TALE proteins have been engineered with catalytic domains of DNA invertase Gin to design new chimeric proteins called as TALE recombinases (TALERs). TALERs have been successfully used in bacteria and mammalian cells and offer an alternate approach to targeted GenEd [126]. DNA-binding domains (DBDs) of hyperactivated variants of the resolvase/invertase family of serine recombinases can be replaced with engineered ZFs to retarget them to sequence of interest in the genome. However, imperfect modularity with particular domains, lack of high-affinity binding to all DNA triplets, and difficulty in construction were major limitations in widespread usage of ZFPs for genome editing. Mercer et al. [126] designed a TALE recombinase (TALER) through engineered fusion of a hyperactivated catalytic domain from the DNA invertase Gin and an optimized TALE protein. The TALER architecture significantly increased the targeting capacity of engineered recombinase as well as its potential applications in plant and animal biotechnology. In cotton, meganucleases were also used for gene stacking based on homologous recombination [37]. TALENS has been described as the most precise technique for targeted gene stacking of economically important molecular traits in crop plants. Cotton genome has been modified efficiently using GenEd tools. Successful reports of GenEd in cotton have been given in **Table 4**.

Genome editing tool	Gene	Gene modification	Reference
Meganucleases	*HPPD*, *EPSPS*	HDR, gene stacking	[27]
CRISPR/Cas9	*GhCLA1*	Multisite GenEd	[62]
CRISPR/Cas9	*GhARG*	NHEJ	[62]
CRISPR/Cas9	*GhMYB25-like* A and D	NHEJ	[102]
CRISPR/Cas9	*GhPDS*, *GhCLA1*, *GhEF1*	GenEd	[123]
CRISPR/Cas9	*GhCLA1*, *GhVP*	NHEJ	[28]
CRISPR/Cas9	*GFP*	NHEJ	[53]

Table 4. Genome editing in cotton.

8. Targeted mutagenesis for functional genomics studies in cotton

GenEd tools are precise and highly specific. For reverse genetics and functional genomics, these reagents are also advantageous over the existing approaches, TILLING. For targeting gene families, TILLING is limited due to high specificity of the primers [127]. TILLING is difficult in polyploidy genomes; further, its low mutation rate and high screening cost make it more limited compared to ENs. CRISPR/Cas9 can target multiple genes simultaneously with multiple gRNAs. For functional genomics in cotton, ENs can be used with higher specificity and low cost. RNAi has been previously used successfully for functional genomics in cotton [128]. RNAi works at the posttranscriptional level and, hence, may lead to off-target, unreliable, and unpredictable results. Moreover, RNAi may also result in induction of unspecific immune response and incompleteness of knockdowns. All these limitations can be overcome using highly specific, more reliable, and less costly GenEd tools. Additionally, ENs work at the transcriptional level; henceforth, are more predictable; and would result in complete knockdown. Multiplexing has further made RGENs more fascinating than any other technique to study gene families and polygenic characters. Chen et al. [28] demonstrated CRISPR/Cas9-based targeted mutagenesis of cotton cloroplastos alterados 1 (GhCLA1) and vacuolar H+-pyrophosphatase (GhVP) genes and confirmed targeted/site-specific single nucleotide insertion and substitution in GhCLA1 and one deletion in GhVP.

Multisite GenEd in cotton has also been reported earlier. Wang et al. [62] utilized a CRISPR/Cas9 system to conduct multisite GenEd in allotetraploid cotton. An exogenous gene DsRED2 and an endogenous gene GhCLA1 were targeted with 66.7–100% efficiency. CRISPR is efficient in multisite GenEd with high successful rate. For gene function studies in cotton, a highly efficient platform has been developed using CRISPR/Cas9 [102]. They used GhMYB25-like gene to study gene knockout mutants in cotton. Moreover, 1–7 nt deletions were observed with one sgRNA, while deletion of 168-nt-long fragment was deleted using two sgRNAs. An efficient and fast method was developed to validate sgRNAs in cotton plant through transient assay. Using this robust method, activity of sgRNAs can be validated in 3 days which will be helpful in selection of potential sgRNAs for stable transformation in cotton. Individual genes (GhPDS, GhCLA1, and GhEF1) were targeted resulting in typical albino phenotypes by inducing mutation in GhCLA1, simultaneous editing of homoeologous genes, and genomic fragment deletions [129]. This kind of studies made a foundation stone for undertaking functional genomics studies in cotton.

9. Delivery of artificial DNA-binding proteins and ENs into plants

Sequence-specific nucleases enable facile editing of higher eukaryotic genomic DNA; however, targeted modification of plant genomes remains challenging due to ineffective methods for delivering reagents for genome engineering to plant cells. Method of delivery of ENs is very important for appropriate expression and optimum results. In animals, delivery of TALEs or TALENs was possible through nucleic acids, mRNA, as well protein [7, 130, 131]. TALEN activity mainly depends upon delivery method, choice of expression vector, and method of transformation used. Conventional plasmids and viral vectors have been used for expression of required proteins inside the cell. ZFNs were delivered using a novel tobacco

rattle virus (TRV)-based expression system and produced non-transgenic mutant plants [132]. ZFNs were transiently expressed into a variety of tissues and cells of intact plants to produce genetically modified plants. Geminivirus-based replicons have also been used for transient expression of sequence-specific nucleases (ZFN, TALENs, and CRISPR/Cas) and delivery of DNA repair templates [133]. In tobacco, the use of viral replicons enhanced gene targeting efficiency by twofolds compared with conventional *Agrobacterium tumefaciens* T-DNA.

Transient expression of the CRISPR/Cas9 ribonucleoprotein complex in protoplasts can result in the production of specifically targeted, transgene-free mutants in the T_0 generation in several plant species [134]. Highly efficient and specific transient expression-based genome-editing system was developed for producing transgene-free and homozygous wheat mutants in the T_0 generation [135]. Genome-edited DNA-free bread wheat was produced using CRISPR/Cas9 ribonucleoproteins (RNPs) [136]. RNPs were delivered into wheat immature embryos through particle bombardment. Cas9 protein was expressed and purified from *Escherichia coli Rosetta* strain, and the sgRNA was transcribed using HiScribe T7 In Vitro Transcription Kit (New England Biolabs). CRISPR/Cas9 RNP-mediated GenEd eliminates the risks of transgene integration into plant genome and further promises targeted gene mutations with no off-targets. Moreover, it is fast and robust compared to other methods.

In case of TALENs, the use of mRNA is advantageous than permanent integration of T-DNA in genome. Firstly, in pharmaceuticals viral vectors are perceived as gene-modified organisms, while mRNA has superior regulatory viewpoints. Secondly, delivery of transient mRNA reduces any risks of unwanted stable integration and mutations in the genome. Gallie [137] introduced mRNA into plant protoplast efficiently using PEG-based transformation. So, the TALEN mRNA delivery could be more attractive for transient expression in plants to avoid undesirable results and to prompt regulatory process. Moreover, in case of nuclease, which introduces double-strand breaks, the integration and continuous expression of the gene into the host may lead to detrimental results. Synthetic mRNAs of TALENs for GenEds are available from different companies like TriLink BioTechnologies at request.

In biomedical industry, direct injection of CRISPR and TALEN proteins in living organisms is very fascinating. Direct delivery of proteins may further reduce the limitations and concerns of posttranscriptional and translational constraints associated with expression of plasmid and mRNA. Direct delivery of purified nuclease proteins was reported in *N. benthamiana* protoplasts using PEG and was claimed as non-transgenic GenEd approach [138]. Direct delivery of EN proteins into plants would be proven as the most favorite approach for regulatory approval of edible crop plants and cotton as well. On the basis of previous reports discussed above, the production of non-transgenic cotton would be very helpful from regulatory and public acceptance viewpoint.

10. Comparison of ENs

All technologies have almost same mode of action and give same results, but these are different from one another in terms of nature, components, target specificity, target requirements, target limitations, modularity, and construction assembly methods. On these bases current GenEd tools are compared in **Table 5**.

Features	ZF(N)s	TALE(N)s	CRISPR/Cas9
Origin	*Xenopus laevis*	*Xanthomonas* (similar proteins also reported in *Ralstonia solanacearum* and *Burkholderia rhizoxinica*)	*Streptococcus pyogenes* (present in 40% bacteria and 90% archaea)
Nature	DNA-binding motifs in eukaryotes	Plant pathogenic protein	Prokaryotic defense protein
Function	DNA binding as transcription factors	DNA binding and gene modulation of host plant (act like transcription factors)	Endonuclease that cuts DNA of infecting viruses and plasmids
Target binding	Protein-DNA (one to triplet)	Protein-DNA (one to one)	RNA-DNA (one to one)
Components	DNA-binding domain	DNA-binding domain	Endonuclease
		Effector domain (activator/repressor)	gRNA
Year of emergence as GenEd tools	2000	2010	2012
Target length	~9–36 nt	~12–50 nt	~20–23 nt
Target limitations	It binds to a triplet of DNA bases	Needs T base at 5′	Needs PAM region (5′NGG)
Modularity	Low	High	High
Off-targeting	Low	Very few	High
Size	Small	Relatively big (small in case of TALEs)	Big
Mode of action	DNA binding and DSB (NHEJ/HR)	DNA binding, expression modulation/ DSB (NHEJ/HR)	DNA binding and DSB (NHEJ/HR)
Assembly	Difficult	Technical but easy	Easy
Uses	Gene disruption, gene deletion, gene correction, gene addition, tag ligation, ObLiGaRe	Gene activation, gene repression, gene disruption, gene deletion, gene correction, gene addition, tag ligation, ObLiGaRe	Gene disruption, gene deletion, gene correction, gene addition
Epigenome editing	Less reported	More reported (natural TFs)	Less reported
Delivery	DNA, mRNA	DNA, mRNA, protein	DNA
Targeting efficiency	Low and variable	High	High
Delivery via viral vector	Easy	Easy	Challenging
Delivery as RNA molecule	Easy	Easy	Challenging
Delivery as protein	Easy	Easy	Challenging

Table 5. Comparison of three popular engineered proteins/nucleases for DNA targeting.

11. Future perspectives

Genome engineering in cotton using ENs will open up new avenues for gene function studies and understanding of complex polygenic metabolic pathways. Improvement in

cotton growth and development with good quality of fiber and seeds can be achieved more precisely using GenEd tools. Some of the reports of GenEd in cotton using ENs reviewed above are enough to demonstrate success of targeted gene modifications in cotton. Moreover, CRISPR/Cas nickases are used for gene replacement and correction, and the use of this technology for replacement of endogenous promoter with exogenous constitutive, inducible, or strong promoter can be helpful in regulation of expression of endogenous gene. This approach could reduce the risks of foreign gene integration into the genome. Furthermore, tuneable, special, and tissue-specific expression of the endogenous genes can be achieved with the insertion of new promoters at place of indigenous promoters. The risks associated with the development of resistance against Bt can be mitigated by gene pyramiding/stacking through ENs. Modification of epigenome marks associated with certain crop parameters such as flowering, fiber quality, and stress resistance can be obtained with fusion of epigenome modifiers with artificial DNA-binding proteins (ZFs, TALEs, and dCas9). In conclusion, genetic improvement in cotton using GenEd toolbox would be helpful in solving prevailing problems and constraints causing decrease in cotton growth, yield, and fiber quality.

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Section 2

Cotton Fiber Types and Properties

Chapter 3

Impact of the Bijective Relationship between Single and Bundle Cotton Fiber's in Cotton Breeding Programs

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Additional information is available at the end of the chapter

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Abstract

In this chapter, we focus on the relationship between fibers' mechanical properties and yarns' ones by studying their relative behavior and the relationship between single and bundle cotton fibers (respectively, dispositions 1 and 2). For this purpose, three different types of cotton fibers were studied. These cottons were chosen from a list of 12 cottons covering a large panel of varieties and physical properties (maturity, fineness, micronaire, length, tenacity, etc.). Classifications per length classes and linear densities were done in order to have more precision and knowledge of cotton fiber behavior. Modeling the creep behavior of single and bundle fibers will help exploring data for the bijective relationship between the two dispositions. Properties evaluated will include elongation, single fibers and bundle tenacities, work of rupture, and so on. Quality of bundle fibers will be a good tool in predicting spinning performances and thus yarn quality.

Keywords: fiber, yarn, single, bundle, length classes, linear densities, modeling

1. Introduction

Cotton fibers are trichrome from plants of the order *Malvales*, the family *Malvaceae*, the tribe *Gossypieae,* and the genus *Gossypium*. There are four domesticated species of cotton sorted by decreasing commercial importance: *G. hirsutum, G. barbadense, G. arboreum*, and *G. herbaceum* [1, 2]. Fiber traits of few cotton species have been shown in **Table 1**.

Cotton fiber's characterization is very complex and depends on the growing and harvesting conditions of the plant. It is very important for cotton breeders to understand the relationships

	% word prod	Distribution	Commercial varieties	Length (mm)	Tensile strength at zero-gauge (cN/Tex)	Linear density (mTex)
G. hirsutum	90	Central America	Upland cottons	25–32 →medium	40	Up to 200
G. barbadense	5–7	South America, Egypt, Soudan, and Peru	Egyptian, Sea Island, and Pima cottons	Superior to 33 →long to extra long-staple cotton	55	100–140
G. arboreum and *G. herbaceum*	3–4	Africa, Asia, and India	Pakistan and India	Inferior to 25 →short fibers	35	300

Table 1. Fiber parameters of *G. hirsutum, G. barbadense, G. arboreum,* and *G. herbaceum* species.

existing between specific fiber properties, overall fiber, and yarn qualities [3]. All of these factors interact and are critical to the development of cottons that can compete in a global market.

Understanding these interactions will allow breeders for using the data more effectively for selecting the best cotton plants for developing a cotton variety with improved yarn quality [3, 4]. For this purpose, following properties of cotton fiber were described.

2. Cotton fiber properties

2.1. Physicochemical properties

The cotton fiber consists of a primary and secondary cell wall [5]. These latter are the key determinant of the cotton fiber growth and development and are primarily composed of cellulose (about 96% of pure cellulose: which is a naturally occurring crystalline carbohydrate polymer), hemicellulose, pectin, lignin, and structural proteins [2].

Numerous studies comprehensively describe the structure and development of cotton fibers [6, 7]. In brief, cotton fibers develop in three phases: initiation, elongation, and maturation through secondary wall thickening. The initiation of cotton fibers begins from the epidermal cells on the ovule surface to the elongation and the development of the primary cell wall. This latter, covered with a cuticle, continues to elongate until reaching the final fiber lengths. The secondary wall, which makes up 90% of fiber weight, consists of cellulose fibrils arranged in a layered helical structure. This layer contributes to the tensile properties of the cotton fiber and gives the final mechanical properties of the fiber. The final stage in the fiber development consists of the removal of moisture, during which the fiber collapses. Fibers are then converted from a cylindrical shape to a twisted ribbon. **Figure 1** shows the changes in fiber length, diameter, and wall thickness during the development of the cotton fiber.

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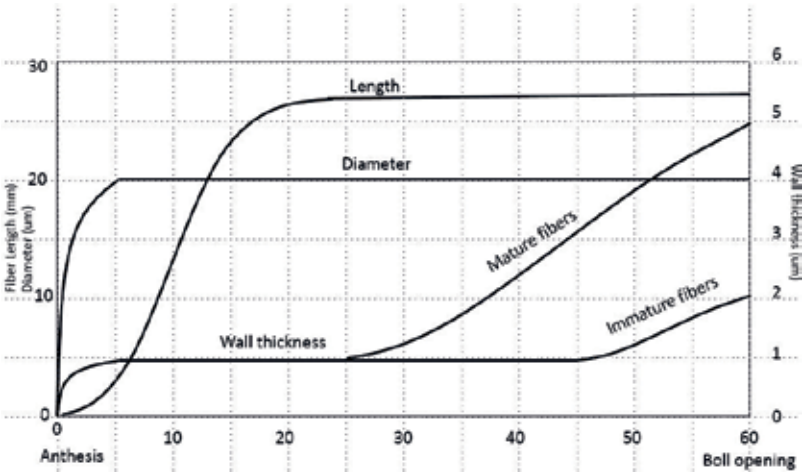

Figure 1. Different developmental stages of fiber.

2.2. Physical properties

2.2.1. Fiber linear density or fineness

Cotton fineness [8–10] is the linear density or weight per unit length of fiber. In fact, for a given fiber (that is assumed of a fixed density), its mass is proportional to its cross-sectional area:

$$\text{Mass of a fiber} = \text{cross} - \text{sectional area} \times \text{length} \times \text{density} \tag{1}$$

This relationship is used in the gravimetric definition of fiber fineness. The primary unit of fiber fineness is Tex (g/1000 m).

2.2.2. Fiber maturity

The maturity [11, 12] of cotton fibers is the degree of thickening, which is defined as the ratio of the area of the cell wall to the area of a circle having the same perimeter as the fiber cross section. Thus, measuring the maturity of cotton fibers involves measuring the thickness of their secondary cell wall. The degree of wall thickening increases as the fiber matures.

Based on microscopic observations, wall thickness can be denoted by the degree of thickening (θ) as shown by the equation below [9]:

$$\theta = \frac{\text{cross} - \text{sectional area of fiber wall}}{\text{area of circle of same perimeter}} \tag{2}$$

When the degree of thickening is equal to one, the fiber is then completely solid. When the value of θ is above 0.6, the fiber is considered mature. In the contrary, when θ is below 0.6, the fiber is considered immature.

2.2.3. Fiber micronaire

Micronaire is an indicator of both fineness and maturity. Its value is determined by the measurement of the air permeability of a mass of cotton fibers under specified conditions. Micronaire [13, 14] measurement fails to properly distinguish fine and mature cotton fibers from coarser with lesser maturity. The empirical relationship combines the maturity, fineness, and micronaire:

$$(\mathrm{MR})^2\, Tt = 3.86\,(IM)^2 + 18.16(IM) + 13 \quad (3)$$

where IM is the micronaire, Tt is the fiber fineness, and MR is the maturity ratio.

2.2.4. Fiber length

Enhancing fiber length is a complex issue because fiber samples from cotton bales contain a range distribution of fiber lengths [15].

The prevailing environmental conditions during a growing season affect the length distribution of cotton fibers [16]. The length of cotton fibers fluctuates significantly not only among cultivars but also within a cultivar. It is because of the prevailing environmental conditions within the same plant due to position of the boll, within the same boll due to the flow of nutrients toward the developing individual seed, and within the same seed due to the positions of fibers on the seed. Besides, harvesting, ginning, and processing methods change the length distributions of cotton [17]. Determining the length of individual fibers is time consuming and difficult, so various methods for estimating fiber length have been devised. Most test methods and instruments for fiber length analysis measure the length and the weight of each group of fibers in order to determine the fiber length characteristics.

2.3. Mechanical properties

Mechanical properties are the most important indicators for breeders to produce fibers that perform better in textile manufacturing and end-user [18]. Fiber mechanics is the study of the tensile, creep, relaxation, and fatigue properties, the key of the mechanical properties, for fibers and fibrous assemblies [19, 20]:

- Tensile test

Tensile test is carried out to achieve fiber parameters such as strength, percent elongation, and initial modulus, and these important parameters are usually obtained after applying axial stress at a constant elongation rate till failure. The applied tensile load and elongation are recorded during the test for the calculation of the stress and the strain.

- Creep test

Creep is a time-dependent deformation under a certain applied load. The rate of deformation is named the creep rate. It is the slope of the line in a creep strain vs. time curve.

- Relaxation test

In the relaxation test, a constant strain is applied, and the stress is measured for a period.

- Fatigue test

In a fatigue test [21], a fiber may be cycled over a wide range of frequencies under a variety of imposed extension rate conditions. The fiber is held between two clamps one of which connected to a vibration generator. An oscillatory force is applied to the fiber and is chosen as a percentage of the breaking force.

3. Measurements of cotton fiber properties

Cotton fibers are not homogeneous in their physical properties. Their maturity, fineness, and lengths vary from fiber to fiber. Sometimes, even alongside the length of a fiber, there is a variation in physical properties. Thus, the mechanical properties are affected by these variabilities.

Table 2 regroups almost all the apparatus allowing to determine the physical and mechanical properties of cotton fibers.

Physical properties	*Fineness*	- Projection microscope
		- Airflow
		- Advanced Fiber Information System (AFIS ®) [22]
		- Vibroscope
	Maturity	- Goldthwait differential dyeing [23]
		- Double compression airflow measurement
		- Polarized light analysis
		- Causticaire
		- Centrifugal methods
		- Image analysis
		- Near-infra-red (NIR) spectrometry
		- X-ray fluorescence spectroscopy
	Micronaire	- Fineness and Maturity Tester (FMT)
		- Fibronaire
		- Cottonscope [24]
		- Advanced Fiber Information System (AFIS ®)
	Length	- Zweigle Sorter
		- WIRA fiber length machine
		- Fibrograph
		- Almeter
		- High Volume Instruments (HVI ®) [23]
		- Advanced Fiber Information System (AFIS ®)

		Single fibers	Bundles
Mechanical properties	*Tensile*	- Mantis Single Fiber Tester [25] - Favimat [26] - Universal Fiber Testing Machine (UFT ®) [20] - Dynamometer MTS	- Stelometer - HVI ® - Dynamometer MTS
	Creep relaxation and fatigue	- Dynamometer MTS - UFT ®	- Dynamometer MTS

Table 2. Apparatus allowing measuring fiber physical and mechanical properties.

4. Case study

4.1. Materials and methods

To determine the bijective relationships between single and bundle cotton fibers, 12 bales of cotton were selected based on their distinct physical properties. These cover a large panel of cotton micronaire, tenacity, and lengths. Prior to testing, all cotton samples were conditioned for at least 48 h at 65 ± 2% RH and 21 ± 1°C.

4.1.1. Physical property measurements

The mean values of fineness, maturity, micronaire, and length were determined by the Fineness Maturity Tester and Micromat. The results are shown in **Figure 2a** and **b**.

The principle of the AFIS consists of single fiber measurements with an opto-electronic sensor. Fiber creates signal/impulse converted to an electrical signal, which is analyzed and evaluated

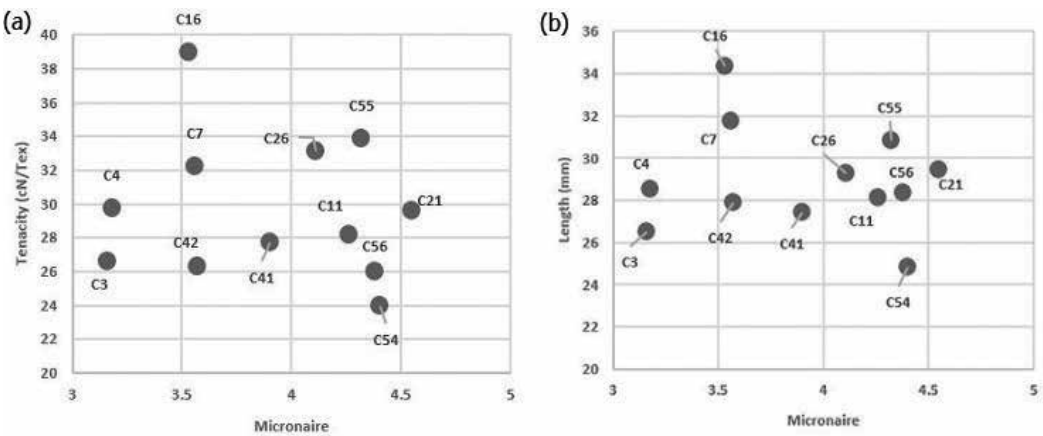

Figure 2. (a) Tenacity vs. micronaire. (b) length vs. micronaire.

by computer. For instance, for length values, the main measurements include: mean length, length upper percentiles, length CV%, short fiber content (defined as the percentage of fibers less than 12.7 mm in length), upper half mean length (UHML), and upper quartile length (UQL).

However, for determining the length, Zweigle sorter method was used. It allows sorting fiber length into groups. It is based on using a Johannsen-Zweigle apparatus (**Figure 3**). The device consists of two steel comb fields to align and straighten the fibers. For cotton sample testing, the combs are spaced from each other for a distance of 4 mm, and the weight of the test specimen is 100 mg. Once prepared, the fibers go through repeated drawing and doubling processes to form straight and parallel bundle of fibers. Length intervals are obtained allowing classification of the fibers into groups. In order to obtain a mass distribution of sample, the fibers of each group are then weighed. The length's interval of each group is determined by the spacing of the combs. We must have at least 10 sample groups extended on the longest fiber. Thus, the longest fibers are drawn and weighed first, followed by the shorter.

4.1.2. Mechanical property measurements

4.1.2.1. Single fiber testing

For measuring single fiber properties, Favimat (Textechno Herbert Stein GmbH and Co. KG, Möchengladbach, Germany; **Figure 4**) was used. The typical testing methods of the Favimat

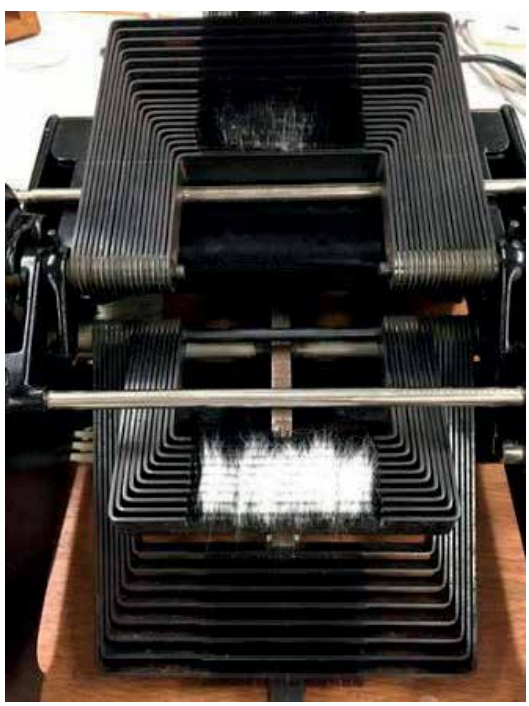

Figure 3. Zweigle sorter apparatus of the Laboratoire de Physique et Mécanique Textile (LPMT).

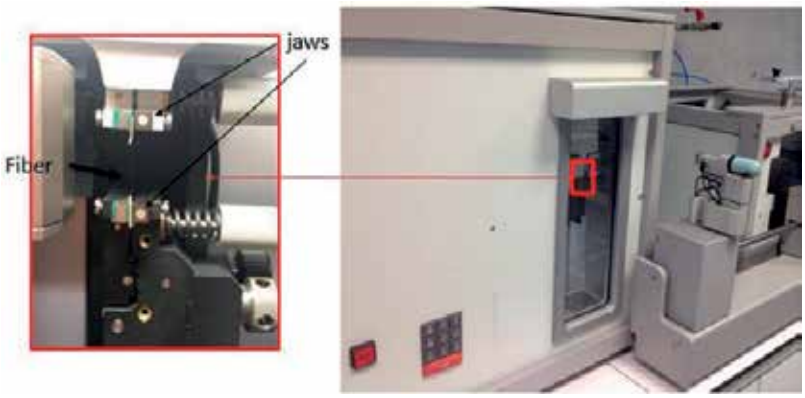

Figure 4. The Favimat device for tensile and linear density measurements.

are the static tensile test, linear-density (fineness) measurement, and measurements of crimp extension, crimp stability, and number of crimps.

The main principle is that both single fiber ends are clamped between two sets of jaws. The displacement is insured by a constant speed motor with interchangeable equipment to vary the rate of elongation. For the tensile tests, the data of the load and elongation are transferred to a computer to be plotted and analyzed.

Universal fiber testing machine (UFT) was also used. This apparatus allows to carry out the testing of single fibers in tensile, creep, relaxation, and fatigue. It was developed by Bunsell and Hearle in the 1970s. **Figure 5** shows the UFT device of the LPMT laboratory.

In addition, the dynamometer MTS with a 2 N sensor was used for the single fiber creep and relaxation tests. The fibers are glued with a cyanoacrylate glue in the extremities of a 15-mm diameter paper. Once placed in the two jaws of the dynamometer MTS (**Figure 6**), paper is cut on its extremities to allow the extension of the fiber.

4.1.2.2. Bundle fiber testing

For bundle testing, Pressley clamps were used and placed on a dynamometer device. An attachment system was compatible with the dynamometer MTS, and the jaws were designed in our laboratory as shown in **Figure 7**. The sensor used for the bundle fiber test had a 2-kN sensibility.

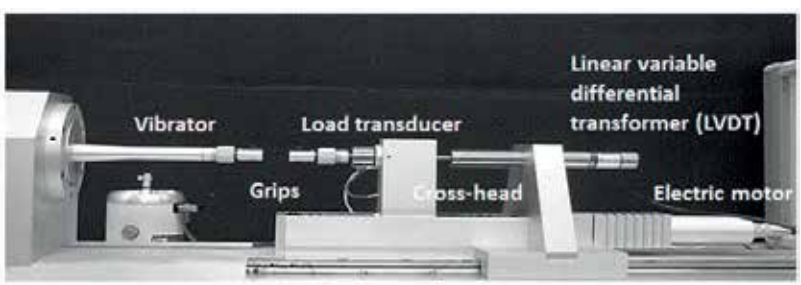

Figure 5. UFT device of the LPMT laboratory.

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Figure 6. Single fiber disposition in the dynamometer MTS.

Preparation of the samples in the Pressley jaws is similar to the one used for the Stelometer measurements. In fact, the specimen is pulled manually through the teeth of a comb several times to straighten fibers and remove all the neps. A flat bundle of fiber is placed in the device (**Figure 8**) and is fixed with in a special vise, which provides a pre-stressing load at a 100-g tension. A torsion spring ensures uniform tightening of the clamps. The ends of the sample are then cut off with a special knife, and finally, the clamps are placed in the instrument.

Figure 7. Pressley clamps in the dynamometer device.

Figure 8. Stelometer device of the LPMT.

4.1.3. Analogical modeling

Tests allowed to determine the physical properties of cotton fibers are good indicators of the global behavior of fiber. In fact, many parameters can be determined from these tests (such as E-initial modulus, σ-stress, ε-strain, η-viscosity, etc.). Analogical modeling can be a way to simulate cotton fiber behaviors, whatever the disposition tested.

Analogical models are represented based on the assembly of simple mechanical elements (springs, dashpots, skidding blocks, or stopping blocks) having the same responses to those expected by the real material. These models are very useful to clarify how the fiber (single or bundle) behaves. The most common elements are shown in **Table 3** [27]. The mechanical elements can be assembled both in series or in parallel or in mixed groups (networks). Thus, more complex mechanical responses can be simulated to illustrate the behavior of the material submitted to the tensile, creep, relaxation, and fatigue tests [21, 28].

Cotton fibers are viscoelastic. Creep and stress relaxation tests demonstrate this characteristic. In creep test, a constant stress is maintained on a specimen while its deformation is monitored as a function of time, and deformation increases with the time. In stress relaxation test, a constant deformation is maintained while the stress on the specimen is monitored as a function of time, and stress decreases with time.

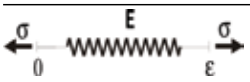
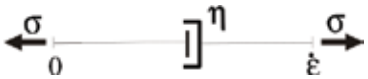
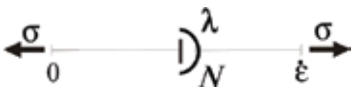
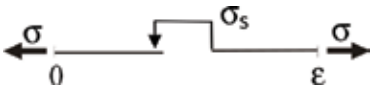
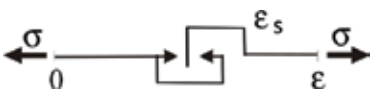
Analogical model	Equation	Mechanical element	General signification
	$\sigma = E\,\varepsilon$ where σ is the stress, ε is the strain, and E is Young's modulus	Spring	Linear elasticity presented by a linear relationship between the stress and the strain, and the model obeys to Hooke's law
	$\sigma = \eta\,\dot{\varepsilon}$ where σ is the stress, $\dot{\varepsilon}$ is the strain rate, and η is the viscous coefficient	Dashpot	Linear viscosity presented by a linear relationship between the stress and the strain rate, and the model obeys to Newton's law
	$\sigma = \lambda\,\dot{\varepsilon}^{1/N}$ where λ is a constant related to the material used and N is a constant characterizing the flow		Non-linear viscosity that depends on the material tested and the flow
	$-\sigma_S < \sigma < \sigma_S$ where σ_S is the stress threshold	Skidding block	Plasticity that depends on the stress threshold
	$-\varepsilon_s < \varepsilon < \varepsilon_s$ where ε_s is the strain threshold	Stopping block	Plasticity that depends on the strain threshold

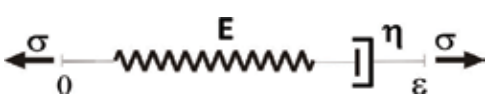
Table 3. Standard linear solid models.

The classical viscoelastic constitutive models are represented by Maxwell and Kelvin-Voigt models using springs and dashpots to simulate elasticity and viscosity, respectively. The respective equations are:

$$\dot{\varepsilon} = \dot{\varepsilon}_{elastic} + \dot{\varepsilon}_{viscous} = \frac{\dot{\sigma}}{E} + \frac{\sigma}{\eta} \tag{4}$$

$$\sigma = \sigma_{elastic} + \sigma_{viscous} = \eta\,\dot{\varepsilon} + E\,\varepsilon \tag{5}$$

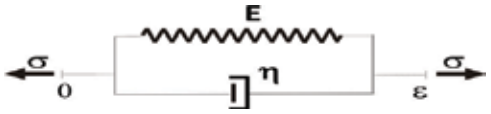
In the case of the Maxwell model, the behavior is modeled by a spring and a dashpot connected in series:



Eq. 6 gives response of the Maxwell model. For example, for the creep and relaxation tests, where a constant strain ($\varepsilon = \varepsilon_0$) and a constant stress ($\sigma = \sigma_0$) at t = 0 are applied, respectively, the respective following responses are obtained:

$$\varepsilon = \frac{\sigma_0}{E} + \frac{\sigma_0}{\eta} t \quad (6)$$

In the Kelvin-Voigt model, the spring and the dashpot are connected in parallel:



The Kelvin-Voigt response of the creep test at a constant stress σ_0 at t = 0 is:

$$\varepsilon(t) = \frac{\sigma_0}{E}\left(1 - e^{\left(-E \cdot \frac{t}{\eta}\right)}\right)$$

4.2. Results

Among these 12 varieties of cotton fibers, C7, C42, and C55 were selected. In fact, C7 and C42 have the same micronaire but showed different tenacities and lengths. However, C7 and C55 showed the same length and tenacity but different micronaires. Classification per length using the Zweigle Sorter was carried out in order to get more information about the variability of tensile properties across various length classes. The classes found for each cotton are shown in **Table 4**.

Single and bundle tests are carried out for each length class of each cotton fiber.

Tensile tests of single fibers can be undertaken with the Favimat, which determines the linear densities. This latter is measured according to the vibroscopic testing principle. Two stickers are attached in extremities of the cotton fibers and placed between the two jaws of the Favimat. Test parameters are as follow, and the test results are shown in **Table 5**:

- Test speed: 5 mm/min.
- Gauge length: 15 mm.
- Sensor: 210 cN.
- Pretension: 0.06 cN/Tex.
- Nominal linear density: 10 dTex.

Creep and relaxation tests of single fibers were done with a dynamometer MTS.

	[38–36]	[36–34]	[34–32]	[32–30]	[30–28]	[28–26]	[26–24]	[24–22]	[22–20]	[20–18]
C7										
C42										
C55										

Table 4. Length classes for cottons C7, C42, and C55 (the shading illustrates the length classes).

	Elong max (%)	Elong rupt (%)	Force max (cN)	Work of rupt (cN cm)	Tenacity (cN/tex)	Linear density (dtex)	Time (s)	Specific modulus E
C7 [38–36]	7.12	7.62	5.34	0.26	94.85	0.58	13.83	13.54
C7 [36–34]	8.16	8.30	5.35	0.30	72.48	0.76	15.04	9.11
C7 [34–32]	7.66	7.83	4.84	0.27	58.18	0.84	14.19	7.81
C7 [32–30]	8.50	8.64	4.78	0.28	62.33	0.78	15.70	7.95
C42 [34–32]	7.23	7.37	4.09	0.21	42.86	1.04	13.40	6.00
C42 [32–30]	9.02	9.17	4.42	0.26	42.28	1.08	16.71	4.96
C55 [36–34]	9.24	9.38	6.90	0.41	99.96	0.71	16.99	11.48
C55 [34–32]	8.04	8.18	5.00	0.29	65.79	1.04	14.88	7.89
C55 [32–30]	7.66	7.80	5.65	0.29	85.05	0.68	14.15	11.36

Table 5. Tensile tests results for some length classes of the cottons C7, C42, and C55.

Test parameters are as follow:

- Test speed: 5 mm/min.
- Gauge length: 15 mm.
- Sensor: 200 cN.

Results (**Figure 9**) showed that fibers behaved similarly during the creep test for the length class [34–32] for the cottons 7, 42, and 55. This result is in coherence with the values of the initial modulus E determined from the tensile tests for the same length class.

We can also note that the strain increased and asymptotically approached the value of $Ln(\sigma_0/E)$ when t tended to infinity. The response of this model to an applied stress is characterized by a fast-increase part explained by the fact that the stress is at first carried entirely by the viscous element. The second part characterized by a very slight increase explains the elastic element in the continuous elongation of the viscous element. The transition time between the two parts represents the creep time constant, t, which is equals to η/E, where η is the viscosity and E is the initial modulus given by the tensile test at a given constant rate of extension [28].

We concluded that the creep response was viscoelastic and therefore that we could apply a Kelvin-Voigt model.

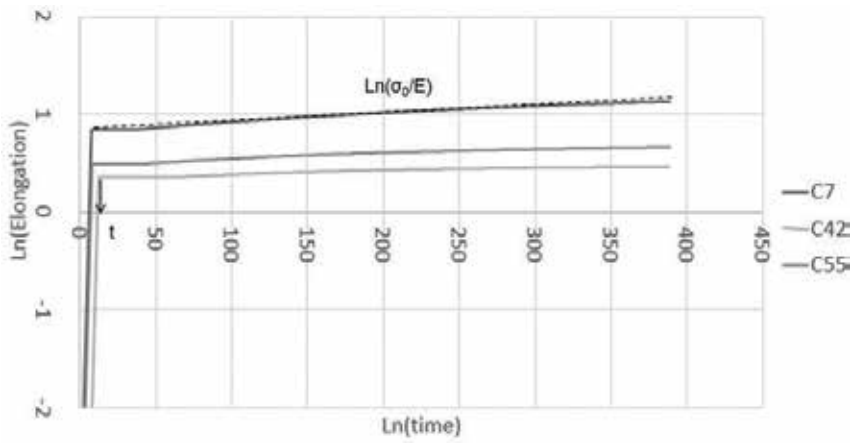


Figure 9. Creep test responses for cottons C7, C42, and C55 for length class [34–32].

As for breeders and geneticists, new cotton varieties developed either by the conventional techniques or by genetic engineering, and detailed characterization of physical and mechanical properties of cotton fibers is essentially required. These properties must be tested fiber-to-fiber, which is very lengthy, tedious, and expensive procedure. Finding a relationship between single and bundle fiber models would therefore be desirable. The aim is then to be able to test these varieties in bundles and to directly determine the single properties.

For bundle testing, the tests were carried out with a speed of 50 mm/min.

Data acquired from the tensile tests are:

- Load and elongation at peak.
- Initial modulus.
- Work of rupture.

The examination of the behavior from the load-elongation curve (**Figure 10**) revealed that we could approximate their shapes to a right-angled triangle, with the base being the elongation and the height being the peak load.

Evaluation of cotton fiber tensile properties serves multiple purposes [29]. The results obtained enable to estimate the performance of raw materials during the transformation procedures of fibers. It is also used to predict the tensile properties of spun yarns or woven textiles. Fiber bundle tensile tests can appear to satisfy the objective because of their relationships with tensile properties of yarn. However, this relationship can be rapidly expected in assemblies of parallel fiber factors such as the degree of fiber-to-fiber interactions and twist contribute to fiber bundle strength.

Regarding creep test results for bundles, we are working on to find out the corresponding models of each cotton variety and comparing them with the single ones. We estimate the bijective relationships existing between the two cotton fiber testing dispositions.

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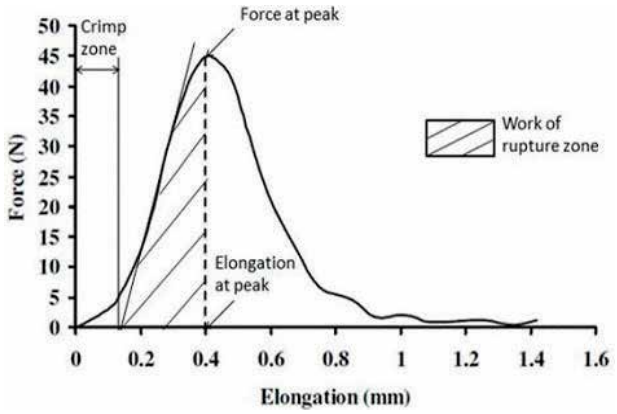

Figure 10. Response of the bundle fibers to tensile tests.

5. Conclusion

Being among the finest natural fibers, cotton is subjected to a variety of characterization tests. These latter can be classified into moisture absorption, electrical, thermal, physical, and mechanical. Cotton breeding programs must deliver fibers that better perform in textile manufacturing in order to compete effectively with international growths of cotton and with the various man-made fibers. In fact, the performance of fibers in textile processing and the quality of the final fabric are highly dependent on the mechanical properties of raw fibers.

Tensile properties of yarns and fabrics depend on both complex fiber arrangements (including length, diameter, friction, etc.) inside the yarn and fabric structure and on the tensile properties of fibers. Thus, it is necessary for the breeders to know about the complex relationships between the fiber arrangements parameters.

In this chapter, we showed that the mechanical behavior of fibers can be analogically modeled and that we assimilate the cotton single fibers creep response to a Kelvin-Voigt one. Nevertheless, the relationship between fiber-to-fiber testing and bundles remains to be clarified through further research.

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Section 3

Genomics of Cotton Traits

Chapter 4

Temperature Extremes in Cotton Production and Mitigation Strategies

Syed Adeel Zafar, Mehmood Ali Noor, Muhammad Ahmed Waqas, Xiukang Wang, Tayyaba Shaheen, Mubashar Raza and Mehboob-Ur-Rahman

Additional information is available at the end of the chapter

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Abstract

Cotton is an important cash crop, providing raw material for different industries and plays crucial role in the economy of several countries. It requires optimum temperature for economic production and causes reduced yield otherwise. Extreme temperature, more importantly, high temperature causes serious yield reduction in cotton by affecting its physiology, biochemistry and quality leading to poor agronomic produce. Freezing temperature also affect the germination percentage and seedling establishment. Several breeding and genomics based studies were conducted to improve the cotton production under high and low temperature stress in cotton. Here we overviewed several agronomic practices to mitigate the effect of extreme temperature, and multiple breeding and molecular approaches to enhance the genetic potential of cotton for temperature tolerance by Marker assisted selection or transgenic approach.

Keywords: cotton, genomics, heat stress, freezing stress, marker assisted selection

1. Introduction

Ever-increasing variability in world climate is threatening the cotton production globally due to temperature extremes, drought stress and irregular rainfall patterns. More than 50% yield reductions in arable crops has been accounted due to these said stresses worldwide [1]. Cotton production is severely affected due to these abiotic and several biotic stresses, thus resulting in

reduced yields and inferior harvest quality. Having indeterminate growth habit, cotton crop bears a complex set of fruiting pattern which is considered to be severely prone to climatic interactions as well as management techniques with differential response [2]. Cotton plant responds to various stresses differently, depending upon the stress severity and the developmental stage. Among various aforementioned stresses, temperature stress in cotton is of key importance as it may cause drastic impact during germination, early growth season, flowering and during the boll formation stages. As the change in global climate is inclined to cause increase in average temperatures, therefore, high temperature may impact cotton crop in the form of longer growing seasons, more or fewer rainfalls, and thus a shorter growing period. Whereas, low temperature during the planting time impairs the seed germination process, oppositely high temperature is also an undesirable feature during planting time. Temperature stress in terms of both cold and heat stress induces a differential metabolic and physiological responses in cotton, through alterations in plant photosynthetic performance, oxidative balance, normal protein synthesis, stomatal closure, membrane damage, lipid peroxidation and carbohydrate production [3–5]. In consequence, various stress responsive mechanisms are triggered by molecular networks to stabilize the internal homeostasis by protecting and repairing of damaged membranes and proteins [6]. Meanwhile, certain heat shock proteins and antioxidant enzymes get activated to combat with induced oxidative and membrane damage within the plant body, resulting in plant tolerance to imposed stress. Still a number of key molecular and physiological mechanisms involved in this homeostasis stabilizing process are under way to find. Here, we discussed the recent advances and understandings in this regard, how the temperature stress affects cotton crop and its induced response by crop plant.

2. Critical stages of cotton development for temperature stress

During late developmental stages, high temperature could lead to increased shedding of flower buds. Boll retention is utmost desirable for higher values of harvests, while high temperatures during this stage severely affects the boll retention, as compared to any other factor. Because high temperature also causes altered boll development (boll size) and maturation period [7]. Similarly, high temperature was also reported to affect the fiber quality in terms of high micronaire values and fiber strength, which are undesirable traits [7]. Low temperature stress, on the other hand, is also devastating during the germination phase of cotton crop as well as for fiber development stage by delayed elongation period and reduced cell wall thickening [8]. Therefore, the stress impact can be categorized in one way, depending upon the severity and duration of temperature stress; on the other hand, the crop growth stage under stress determines the ability of crop plant to tolerate the imposed stress. There is substantial data reporting severe yield reduction under heat stress during late reproductive stages of flowering and boll formation, thus signifying flowering stage as most critical to heat stress [9] along with stand establishment, boll formation and fiber development stages. Pollen development, pollen tube growth, and fertilization are postulated to be the most heat-sensitive stages of the reproductive growth phase in cotton [10].

3. Combined effects of heat and drought are enhanced in plants

Usually heat stress is coupled with limited water availability in many areas of the world. Combined effects of heat and drought stresses are not very widely discovered in cotton, although studied independently. Even though combined effects have been studied in various plants including wheat (*Triticum aestivum* L.) [11], sorghum (*Sorghum bicolor* L.) [12], grasses [13], tobacco (*Nicotiana tabacum* L.) [14], *A. thaliana* [15, 16], maize [17] and tomato [18].

It was observed that high temperatures and water limitations in combination have additive effects of individual stresses. Fundamentally, combination of both stresses aggravates the effects of individual stress. HSPs, reactive oxygen intermediate removal enzymes and many other transcripts were more actively expressed under both drought and heat stress as compared to individual stresses, when examined via transcriptome analysis [19–21]. The same mechanisms involved in response to a single stress are raised under the combined stress. The most promising result of a study conducted in Arabidopsis by Vile et al. [16] was finding a genetic variation of being greatly tolerant to the combined-stress [14].

In a study in cotton (*Gossypium barbadense* L.) by Carmo-Silva et al. [22] it was revealed that the combination of heat and drought stress adversely affect the physiological processes including growth and development compared to single stress. Cotton breeding programs need to focus on selection under both drought and heat stresses instead of focusing these stresses individually [23].

4. Effects of temperature stress

4.1. Agronomy

Although cotton crop originated from warm-climate, the optimal temperature to accumulate biomass estimated 20–30°C [24]. Likewise, optimal window of temperature for ideal functioning of metabolism and associated enzyme should be 23.5–32°C. Exposure to high temperature (>32°C) limits the growth and development of cotton [25]. Generally, all growth stages are affected by high temperature but reproductive stage is the most sensitive and critical one. High temperature reduced the growth period and drastically impacted the agronomical aspects particularly of early maturing varieties [26]. Heat stress reduced the plant height, internodes, sympodial branches, monopodial branches, seeds per boll, boll weight, and fiber length during boll developmental process [27] depending on temperature intensity and exposure period. Suboptimal temperature significantly limited the yield formation process and decreased the boll retention. For instance, an increase of even 1°C in field than optimal-ambient temperatures, lint yield reduced by 110 kg ha^{-1} [28]. This decline in lint yield is principally caused by a smaller boll biomass and low number of seeds produced in a boll [29] by heat-induced pollen damage and low fertility [30] and fertilization efficiency [30, 31]. Recently, Shakoor et al. [32] found that heat stress also limited the uptake of macro and micro nutrients [33].

Exposure to low average and cool night temperature (below 22°C) for extended period is also detrimental for cotton growth. Boll biomass was reported the most vulnerable yield constituent to low temperature because of late-maturity and low availability of carbohydrates induced by late planting of cotton plants [34].

4.2. Physiology

Temperature stress, especially the heat stress, is considered to induce a wide number of physiological and biochemical alterations within the plant cells [3]. It has been observed that mostly the heat stress is coupled with water deficit conditions, thus by causing server injuries to plant cell membrane, disturbed protein synthesis and affecting the photosynthetic apparatus efficiency by reducing the transpiration due to stomata closure [4]. In response of this imbalanced metabolism due to induced heat stress, plants' antioxidative defense system and biosynthesis of a number of new proteins referred to as heat shock proteins (HSPs) get activated to protect plant from oxidative and membrane damage at sub-optimal temperatures [35]. Besides these prominent effects, much of other metabolic and physiological complexities such as chlorophyll synthesis, reproductive efficiency, pollination, fertilization, fiber development, carbohydrate accumulation, reduced water contents, disturbed enzymatic activities, leaf turgor pressure, water transpiration efficiency, fiber strength, fiber elongation time and fruit shedding occur in way due to a substantial increase above optimal temperatures [5, 27, 30, 31, 34, 36–46]. Being originated as hot climate crop, the ideal temperature for cotton plant growth and development lies between 20 and 32°C [35, 47–51]. Optimum performance of cotton crop in terms of maximum number of bolls and square formation, and metabolic activity is reported to occur at day and night temperatures of 30 and 22°C, respectively [49, 52]. A significant decrease in boll retention was observed by Zhao et al. [53] at high temperatures. Burke et al. [36] described the optimum temperature for pollen germination as 28°C and surges above this value is regarded as highly sensitive.

Heat stress at 40°C is reported to cause significant reductions in photosynthetic pigments, proline contents and total soluble sugars along with decreased morphological attributes in two Egyptian cotton genotypes [35]. Moreover, significant variations in number, intensity and density of SDS protein patterns were also observed for said genotypes (Giza 80 and Giza 90). Chlorophyll fluorescence is reported to be lowered at significant levels under high temperature stress or upto 35°C [54]. Photosystem II (PS-II) is regarded as the most sensitive site of the photosynthetic apparatus sensitive to heat stress, while the CO_2 fixation is also considered to be affected at high temperatures [55]. Rubisco activity is also reported to be affected by high temperature stress by suppressing the Rubisco activase enzyme [56–58]. High night temperatures also reduce the fiber micronaire value along with shorter fibers, whereas low night temperatures cause reductions in total cellulose synthesis and hampered boll development [59, 60]. Recently Lauxen et al. [61] observed a critical reductions in seedlings germination potential, growth and the chlorophyll contents under low (18°C) and high (35°C) temperature stress along with different levels of water availability stress.

High temperature stress is reported to affect the pollen viability and the anther indehiscence, resulting in lower seed setting rate and causing significant reductions in final yields [62]. It is

extensively documented and believed that the most viable site to be attacked during the heat spells is the photosynthetic apparatus, which is the primary site for carbohydrate production and food supply to other plant parts. The optimum value for favorable temperatures is considered as 30°C, beyond which the rise in each degree is undesirable. Schuster and Monson [63] proposed a indirect relation between the high temperature and the photosynthetic activity. Because during the stress Rubisco activity is inhibited by protecting the PS-II at high temperatures of 40°C [57]. Seedling stage is also prone to be influenced by temperatures stress in cotton plants possibly due to low germination percentage, fresh and dry shoot weights, turgor pressure, leaf soluble proteins, leaf amino acids and wax contents of epicuticle during the emergence period and early stand establishment [23, 64]. The reason for decline might be the reduced assimilated carbohydrates to newly growing tissues, which was confirmed earlier by the findings of Snider et al. [31] where a decline was observed for carbohydrate translocation to flowers from subtending leaves under stress (heat) conditions. It was also observed that heat stress tolerant cultivars exhibit the higher level of antioxidant activities prior to stress conditions as compared to susceptible cotton cultivars [30].

Declining temperatures and low light intensity due to late planting of cotton crop is attributed to the reduced yield components (boll weight and boll number), reduced fiber elongation rates, and fiber strength mainly due to lower cellulose contents and biomass accumulation [34]. Recently in Australia, Luo et al. [45], proposed a temperature modeling approach and they found that low temperatures will harm less during the early growth with delayed growing season period, whereas the impact of high temperature will be drastic to cotton crop growth, with accelerated crop development especially during the boll formation stage, which can only be catered through management options. Broughton et al. [65] observed the cotton growth and physiological response under elevated CO_2 and temperatures and their combinations, elevated CO_2 caused increase in biomass and photosynthesis, with decreased stomatal activity at ambient temperatures, however these alterations were not evident for elevated temperature. High temperature caused a significant increase in whole-plant water loss (regardless of CO_2 levels) thus reducing whole-plant water use efficiency Broughton et al. [65]. In a recent review by Korres et al. [66], they proposed the implications for elevated atmospheric CO_2 levels by analyzing that positive effects of increased CO_2 on C3 crops may offset the competition for C4 weeds in C3 crops, contrastingly the C3 weeds may threaten the survival of C3 and C4 crops in tropical areas. Elevated night time temperatures cause significant increase in rate of respiration and in response there is reduced carbohydrate accumulation occurs in cotton plants [25, 67]. Pettigrew [68] evaluated six cotton genotypes for their variation in photosynthetic efficiency and heat tolerance and found a very little variation among the genotypes grown in field conditions in a very natural way of inducing heat stress with mild effects, among which only a few lines were observed with reduced (15%) photosynthetic rates. In another study conducted on 16 cotton cultivars, hypocotyl dry weight, leaf pigments and cellular respiration was found affected by heat stress at different developmental stages [69]. Ahmad et al. [70] observed a delay in reproductive stage initiation and accumulated higher thermal time in late maturing varieties as compared to shorter duration cultivars, by sowing at different thermal times, which also decreased the heat use efficiency of seed cotton yield. Alterations in plant water relations, chlorophyll pigments and antioxidant enzyme activities were reported

recently under high temperature conditions (44–46°C) during square and flower initiation stages [71]. In contrast, low temperature stress (15–20°C) caused significant reductions in photosynthetic rates (37%), stomatal conductance (71%), transpiration rate (52%) and intercellular CO_2 (60%), combined with flooding stress in transgenic Bt cotton [72]. Similar decrease in aforesaid physiological parameters were also observed for upland and Pima cotton, when plants employed to combined drought and heat stress, where the maximum decrease in parameters were observed at 35°C [73]. Whereas, high temperatures were also associated with high water use efficiency for both cotton species, with decreased chlorophyll *a* content and improved PS-II quantum efficiency [73]. High temperatures (~35°C) shortened the fiber rapid elongation period significantly, thus reducing final fiber length [74]. A graphical representation of high temperature impact on different growth stages of cotton is shown in **Figure 1**.

4.3. Biochemistry

Effects of abnormal temperature on cotton crop are more pronounced during the reproductive stages namely the boll formation and fiber development. Fiber length, uniformity, strength and the micronaire values are affected by high daytime temperatures, thus affecting the fiber quality [75]. Whereas, the optimal temperature (night) for fiber elongation was proposed

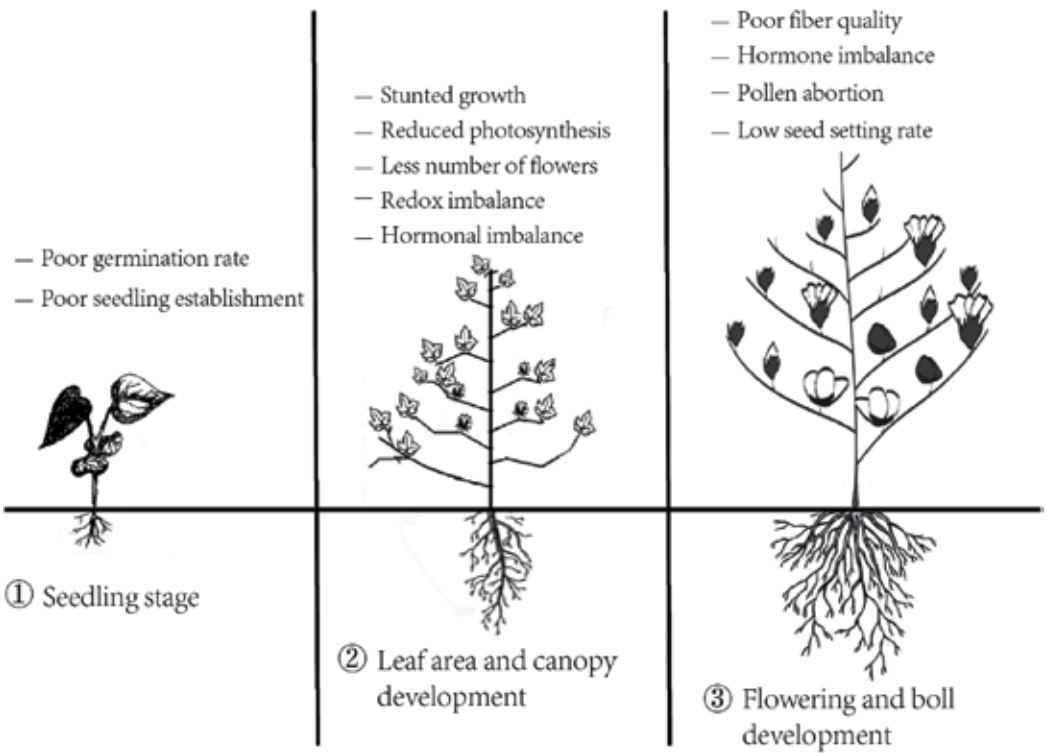

Figure 1. Effect of high temperature on agronomic and physiological attributes of cotton at various developmental stages.

between the range of 15 and 21°C [76]. Major osmotically active solutes in the cotton fiber includes soluble sugars, malate and potassium (K^+), contributing the 80% of fiber sap [77–79], and these components are extensively reported to be influenced by suboptimal temperatures. Moreover, carbohydrate assimilation during boll development in cotton plant is primarily (> 60%) comes from the subtending leaf of boll [80], and this leaf also influenced badly during the hot spells of temperature and drought stress, thus affecting the photosynthetic rate which ultimately imbalances the carbohydrate production in leaf [31]. Recently Chen et al. [81] observed that high temperatures combined with waterlogging conditions inhibits the cell elongation due to influenced osmolyte composition in a newly developing fiber of cotton crop. Further they also confirmed that reduced fiber elongation occurred by alterations in the osmotically active solutes, sucrose, malate and K+ present in fiber sap, which mainly due to waterlogging conditions [81]. Whereas, the high temperatures (34.1/29.0°C) accelerated the early fiber development with reduced fiber elongation periods, mainly due to the altered fiber sucrose content by expression of sucrose transporter gene *GhSUT-1* [81]. Similarly, several genes are reported to induce the anther indehiscence, among which only a few (5 genes) are able to control the carbohydrate metabolism and programmed cell death [82].

Temperature stress may cause deleterious effects at cell and molecular level, their networking and also during protein synthesis [46, 83]. Due to which a number of cellular abnormalities, metabolic imbalances, instable homeostasis and complex molecular reprogramming can be observed at transcriptional and post-transcriptional levels [62]. As we described earlier that heat stress is normally taken together with water deficit (drought) conditions normally in cotton plants thus fortifying the stress impact from both abiotic sources. Sarwar et al. [42] recently confirmed the accumulation of HSPs in response to drought conditions in transgenic cotton containing the HSP gene (*GHSP26*), as compared to wild type. They also observed increased level of leaf water contents (69%), and physiological attributes (photosynthesis, stomatal conductance, transpiration rates and osmotic potential) in the transgenic cotton plants [42]. Cotton genotypes under heat stress, during their evaluation for stress tolerance, are reported to induce the expression of certain HSPs in tolerant genotypes as compared to the susceptible ones [35, 64, 84]. Recently, Wang et al. [85] characterized a cotton abiotic stress inducible TPS gene *GhTPS11,* the over expression of which increased the sensitivity of transgenic *Arabidopsis* seeds under low temperature stress which resulted in increased level of T6P or trehalose. Tolerance of 58 cotton genotypes were assessed for heat stress recently in Pakistan based on some agronomic and physiological parameters, and it was observed that genotypes showed variations to heat tolerance on the basis of affected relative cell injury percentage and heat susceptibility index [86], thus confirming the plausible damage to cell membrane due to stress. Cell membrane thermo-stability (CMT) was proposed by Sullivan [80] as distinct criteria for heat stress assessment. CMT was significantly reduced under high temperature stress ranging from 44 to 49°C as compared to normal field temperatures (37–39°C) in a Pakistani cultivar MNH-886 during 2013–2014 [87]. Iqbal et al. [26] recently evaluated some genes responsible for drought (four) and heat stress [76] in field grown cotton for MAS, they did not found any variations for studied genes responsible for heat stress among the genotypes, thus recommended to include both traits (heat and drought) for selection. Chlorophyll contents and PS-II potential photochemical conversion efficiency of top fourth leaf decreased with increasing

ground water-table and high temperature, along with significant alterations in SOD, POD, CAT and MDA activities due to heat stress at flowering and boll formation stages in cotton [88]. Song et al. [89] have identified sensitive stages of square development at high temperature upto 40°C, they observed the inhibition of pollen tube growth was more pronounced at temperatures above than 35°C, which adversely affected the cotton yield due to heat stress at square development stage. They confirmed that stages from sporogenous cell to tetrad stage (square length < 6.0 mm) was much susceptible to heat stress Song et al. [89]. Snider et al. [90] described that ability to tolerate heat stress could be influenced by plant developmental stages, irrespective of any heat or drought stress, as they characterized this phenomenon for *Gossypium hirsutum* by evaluating PS-II quantum yield, its efficiency and quantum yield of electron transport. Wang et al. [91] suggested that brief water logging conditions with elevated temperatures can improve sucrose composition and its accumulation in subtending leaf, mainly by improved photosynthesis and inhibition of sucrose degradation. The defensive system of a moderately tolerant cotton cultivar from Pakistan could not protect cellular membrane of stressed plants under extreme temperatures (38 and 45°C) [41]. Recently in Pakistan, Khan and his coworkers [92] have screened out some cotton cultivars/lines for heat stress tolerance, and they found significant variation among the genotypes for the evaluating criteria of relative cell injury percentage. Increased night temperatures (30°C) were reported to increase the pistil glucose, sucrose and starch concentrations, whereas the leaf starch concentrations were reduced, which [91] was seen protected by efficient leaf antioxidant metabolism [93]. Besides the deleterious effects of high temperature stress on physiological and biochemical aspects, the chilling temperatures (below 20/15°C) also causes significant alterations and oxidative damages to cotton plant cellular and molecular mechanisms, the extensive review for which (chilling stress) is recently published by Holaday et al. [94], in which the authors described in detail the prominent effects on cotton photosynthetic apparatus and its networking complex metabolism pathways. A pictorial representation of mechanisms of temperature stress tolerance or susceptibility is shown in **Figure 2**.

4.4. Quality

Suboptimal temperature occurrence for few days may affect the cotton yield quality during any time of the growing season. Under stress like excessive heat or moisture, low temperature or nutrients than optimal requirements, cotton shed some squares, flowers, and bolls to ensure survival under unfavorable conditions which caused a significant decline in fiber quality [95]. Cotton yield and fiber quality related aspects (fiber strength, elongation, fineness, and micronaire value) negatively impacted under higher temperature [29]. Although, all stages of fiber formation are affected by temperature extremes, Initial fiber elongation period is most vulnerable to temperature stress. Fiber properties are dependent on photosynthates present in fiber cell walls which are vulnerable to fluctuations in temperature [68]. Suboptimal temperature generally impedes the cellulose synthesis process, and therefore fiber elongation and maturity, consequently, fiber of poor quality is produced [25]. Optimal temperature for fiber uniformity and micronaire was recognized 26°C, and reduced at higher temperature. Moreover, Optimal temperature for fiber length was recognized 18–22°C, and reduced at higher temperatures [96]. Fiber quality is also constrained by low temperature in several cotton-growing regions [97].

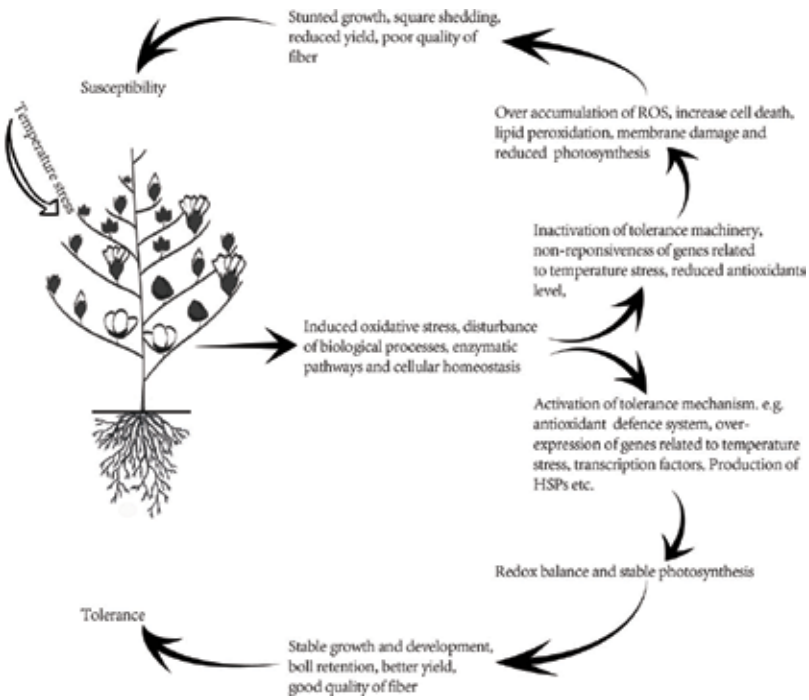

Figure 2. Physiological and biochemical mechanisms of tolerance or susceptibility under temperature stress in cotton.

Exposure to average daily temperature (20.6°C) at fiber elongation stage significantly reduced the fiber quality by changing the expression of proteins involved in cell wall loosening and biosynthesis, osmotic adjustment, and cytoskeleton homeostasis [98].

5. Mitigation strategies to avoid harmful effects of temperature stress

5.1. Agronomic practices

To adopt temperature stress, strategies should be applied according to site-specific conditions. Like, growing the verities of thick cuticle and waxy surfaces that can reflect solar radiation to reduce the impact of heat stress [99]. However, most of the verities are good absorber of solar-radiations, which can increase the stress impact. Recently, it was reported that night temperatures would increase further in future [100] that could adversely affect the cotton productivity. Higher temperatures also limited the cotton growth and development by inducing directs impacts of heat stress, and indirectly by exposing plant to drought conditions. By altering row-spacing under rain-fed systems can increase availability of soil water for plants, impact the lint yield, increase fiber quality, and reduce the level of unpredictability associated with production under stress [101]. Irrigation scheduling based on plant-needs accessed with canopy temperature sensors can also play a crucial role in ameliorating the negative impact of temperature and drought stress [102].

Planting time adjustment is most crucial strategy to addressing temperature stress. Recent finding suggested that changes in planting time significantly affect the cotton growth, lint yield, efficacy of nitrogen utilization and assimilate supply to reproductive organs [92]. Altering planting time would have minor impact on cotton yield irrigated farming systems, but substantial influences on cotton yield of rain-fed farming systems [103]. Adjustment of planting time therefore can ameliorate the negative impacts of stress by adjusting it according to specific growing regions.

Exogenous application of natural and synthetic plant growth regulators [104] is an important and quick agronomic approach to reduce the negative impact of temperature stress [104]. PGRs (Hydrogen peroxide, ascorbic acid, salicylic acid, Moringa leaf extract) significantly enhanced the cotton yield under heat stress by potentiating the cell membranes and enhancing the antioxidant defense [41]. Likewise, exogenous application of benzoic acid improved the cotton performance exposed to heat stress by enhancing the growth rate and nutrients uptake [32].

5.2. Genomic approaches

5.2.1. Marker-assisted selection and identification of QTLs for crop improvement

Molecular marker-assisted selection (MAS) is preferred over visual selection because it is time and cost effective. MAS is a powerful strategy to accelerate the crop breeding for tolerance against biotic and abiotic stresses [105, 106]. Study and development of molecular markers that are linked to the chosen traits [107, 108] and utilization of indirect selection of required loci using molecular markers is a proficient selection tool. Numerous markers have been developed in the recent past like restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), and simple sequence repeats (SSRs) to be utilized in breeding programs via MAS [106].

Usually molecular markers are not developed from the desired genes. On the other hand, development of functional markers (FMs) is generally based on observed polymorphism in transcribed regions of the functional target genes, which make these markers suitable to develop a complete correlation with gene function. Functional markers enable precise selection of target genes [109–111]. However, utility of molecular markers for subsidiary selection is restrained for improvement of traits with marker-assisted backcrossing (MABC) of key genes [112]. Abiotic stress tolerance being the quantitatively inherited traits, which implicates introgression of many genes, is logically not feasible for MAS in breeding programs [113, 114]. In Addition, the requisite of mapping important marker–trait relations through breeding pools, in contrasting environments first, or selection for numerous cycles is another disadvantage of MAS approaches. Marker-assisted recurrent selection [101] approach is a recent strategy comprising of several cycles of the subsidiary selection, is promising to achieve the required occurrence of alleles of target quantitative trait locus (QTL) [115].

Moreover, another modern approach is genome-wide selection (GS) which utilizes the collective influence of genome-wide markers on a trait, which leads to pyramiding promising alleles for minor-effect QTLs [115–117]. Previous knowledge about QTL regulating the required trait is not required, which is the foremost benefit of GS.

Single nucleotide polymorphisms [110], new-generation markers, are abundant, robust and cost effective, are preferred over the conventional molecular markers [118]. Furthermore, these markers can be automated and can competently screen huge populations [119]. Research to identify SNPs for establishment of functional SNPs for prior selection, and for development of high-resolution SNP chips using deep sequencing for association genetics studies is going very fast [118, 120, 121]. High-throughput genotyping of markers and the accessibility of economical, next-generation sequencing platform can effectively facilitate genome-wide selection for crop enhancement in the near future [116, 122, 123]. Recently Fu et al. [124] made an endeavor to develop a substitute for conventional genomic selection using function-associated specific trait FAST SNP markers that can be utilized to accomplish trait-specific prediction more precisely. Continual work to establish better options will lead to improved marker-based evaluations for quantitative traits in molecular plant breeding.

Complex genome of allotetraploid cotton (*G. hirsutum* L.) and its narrow genetic base needs exhaustive work to obtain necessary polymorphism for marker based breeding. In cotton, high throughput markers can be developed utilizing the sequenced cotton genomes coupled with next generation sequencing (NGS) technologies. The perceptions of MAS, QTL mapping and genetic diversity have been coined into genomic selection, linkage disequilibrium and association mapping respectively [125].

5.2.2. Examples of developing markers linked to temperature extremes for MAS in cotton

Mohamed and Abdel-Hamid [35] observed the influence of heat stress at morphological, biochemical and molecular levels in four cotton (*Gossypium hirsutum* L.) genotypes when grown at 30°C for control plants and at 40°C for heat stress treatment. Plants under stress treatment shown a significant impact of heat stress on morphological traits, on the number and intensity of protein bands and activity of isozymes as compared to control plants. This data coupled with RAPD analysis shown two genotypes (Giza 85 and Giza 92) as tolerant genotypes which can be introduced in breeding programs [35].

CAPS and dCAPS the SNP markers developed from specific genes are helpful in molecular breeding of crops. *G. hirsutum* and *G. barbadense* (cultivated allotetraploid cotton species) have discrete fiber quality and many agronomic traits. Kushanov et al. [126] performed the examination and characterization of GSTs of the HY5, PHYA1and PHYB genes of *G. hirsutum* and *G. barbadense* by comparative analysis. They developed one HY5-specific Hinf I dCAPS, one PHYA1-specific Mbo I/Dpn II CAPS and one PHYB-specific Alu I dCAPS cotton markers. These markers could distinguish the two allotetraploid genomes (AD1 and AD2) successfully when tested in parental genotypes of 'Pima 3–79', 'Texas Marker-1' ('TM-1') and their F1 hybrids. PHYA1 gene was mapped on chromosome 11 of A-sub-genome, PHYB gene on chromosome 10 of A-sub-genome, and HY5 gene on chromosome 24 of D-sub-genome, on the reference 'TM-1' x'Pima 3–79' RIL genetic map. The genetic linkage map region containing HY5 and phytochrome-specific markers were found linked with key fiber quality and flowering time traits. In previous studies Kim et al. [127] found the Phytochrome B as a key photoreceptor governing the initiation of cold-stress signaling in light response. These gene markers are valuable candidates in marker-assisted selection (MAS) programs to promptly

introgress *G. barbadense* phytochromes and/or HY5 gene (s) into *G. hirsutum* cotton genotypes or vice versa [126].

5.3. QTL mapping for heat tolerance in cotton

5.3.1. QTLs for heat and drought tolerance

Deriving a connection between a genotype and phenotype is very challenging in the environmental context. Scrutinizing the variations in compound traits either by identifying QTLs in a population developed by crossing two parents or through a genome-wide association study (GWAS) conducted on a set of diverse and distinct individuals, is mainly aimed for identification of alleles responsible for variation in a concerned phenotype. Therefore, studying QTL is vital for recognition of desired genomic regions that can be utilized in molecular breeding programs for improving cotton genetically.

In a study conducted by Ulloa et al. [128], two QTLs were identified for stomatal conductance under high temperatures and irrigated field conditions. Enhanced stomatal conductance provides a cooling effect and in that way, a sort of heat escaping mechanism thus mitigating losses in yield. These findings can be helpful in investigating genetic elements to enhance cotton productivity in warm and dry environments. Studies for the identification of QTLs related with a combination of abiotic stresses are very meager; however, field-based studies relevant to attaining tolerance in field conditions must be emphasized [23].

Certainly, cotton is grown under both elevated temperatures and water shortage. It is also happening because of climate changes globally. Development of varieties, which are tolerant to drought and heat stresses in combination, should be considered by breeders. In a study by Dabbert [129] 138 QTLs for two agronomic and six fiber traits were identified in three separate experiments. Heat sensitive parents were found to have high number of beneficial alleles controlling lint yield and seed cotton yield rather than the heat-tolerant parents. Nonetheless, for polygenic traits a less number of QTLs can be identified in small mapping populations. For the development of tolerant varieties against combined drought and heat stress genomic selection is more practicable in cotton [129].

5.3.2. QTLs for freezing tolerance

Although QTLs linked to low temperature tolerance have been reported in many plants like tomato [130], Rice [131], wheat [33]; However in cotton studies related to identification of QTLs for freezing tolerance are scanty.

5.4. Identification of genes responsive to temperature extremes

5.4.1. Genes for heat tolerance

Possibly, identification of genes for improved yield is the best choice for yield enhancement under optimum production conditions. Under stress conditions those plant perform better which were growing well under high inputs environment [132]. Many studies have been conducted to identify genes involved in tolerance to temperature extremes (**Table 1**) [88].

Identified gene/transcripts	Involvement in abiotic stress tolerance	Species used	Reference
GhDREB1	Cold stress response (transformed into tobacco)	(*G. hirsutum*)	Shan et al. [138]
2 Phospholipase Da (PLDa) genes	Responsive to cold stress	(*G. hirsutum*)	Kargiotidou et al. [137]
GhTIP1	Cold tolerance	(*G. hirsutum*)	Li et al. [136]
GhAGP31	Cold tolerance	(*G. hirsutum*)	Gong et al. [139]
25 ESTs	FPGS3, GhHS126 and GhHS128, responsive to high temperature	Heat susceptible (Nazilli 84S) and tolerant (Stoneville 453, BA 119) cultivars (*G. hirsutum*)	Demirel et al. [133]
94 Heat Shock Protein 20 encoding genes	16 GhHsp20 genes induced with heat stress, and eight genes upregulated by combined abiotic stresses and phytohormone usages	(*G. hirsutum*)	Ma et al. [132]
miRNA encoding genes	319 known miRNAs and 800 unique miRNAs were recognized, and 168 miRNAs were expressed differentially among different temperature treatments	(*G. hirsutum*)	Wang et al. [85]
Heat stress transcription Factors HSFA2, HSFA1b Heat shock proteins GHSP26, HSP101, HSC70–1 encoding genes	Heat stress	Heat-sensitive (ST213 and ST4288) and heat-tolerant (VH260 and MNH456) genotypes of cotton in *G. hirsutum*	Zhang et al. [134]
HY5, PHYA1 and PHYB genes (CAPS and dCAPS markers development from GSTs of the genes)	Cold-stress signaling in response to light	*G. hirsutum* and *G. barbadense*	Kushanov et al. [126]

Table 1. Genes identified in cotton involved in tolerance to temperature extremes.

In cotton cultivars, dissecting the genetic pathways of heat stress responses can help in establishing heat tolerance. Demirel et al. [133] made an effort to determine genes, which were showing response to heat stress in cotton. They used susceptible (Nazilli 84S) and tolerant (Stoneville 453, BA 119) cultivars and sequences of 25 expressed sequence tags (ESTs) were considered for gene homology. Remarkable homology with known genes was found for 16 ESTs, while 8 ESTs were similar to cDNA clones which were not annotated and 1 EST was not showing similarity to any well-known gene. IAA-ala hydrolase (IAR3) and quantitative real-time PCR analysis of the genes revealed that folylpolyglutamate synthase (FPGS3), and two ESTs (GhHS126 and GhHS128) which were not annotated were constantly up-regulated under short- and long-term both heat stresses. The ESTs can be further utilized in developing and enhancing heat tolerance in cotton and other plants. Furthermore, GhHS126 and GhHS128 ESTs can be part of the new favorable genes for heat tolerance [133].

Heat Shock Protein 20 [132] is important for growth and development under abiotic stresses in higher plants. Ma et al. [132] identified 94 GhHsp20 genes in *G. hirsutum*, and clustered them

in 14 subfamilies phylogenetically. Eighty-two GhHsp20 genes were being expressed in at least one examined tissues, which revealed that the GhHsp20 genes contribute in physiological processes and growth in cotton. Two third of the genes were found involved in heat stress response whereas compound stresses induced other 15 genes. The qRT-PCR analysis inveterate the induction of 16 GhHsp20 genes with heat stress, and upregulation of eight genes by combined abiotic stresses and phytohormone usages was confirmed [132].

The endogenous miRNAs, which are a type of sRNAs are involved in transcriptional and post-transcriptional regulation in plants during development and adjective responses to stresses. In response to abiotic stresses including drought, salt, heat, cold, and oxidative stresses, mi RNAs are found to be under or over expressed. MicroRNAs (miRNAs) are a type of non-coding, endogenous RNAs, which control the specific gene's expression by degradation of RNA or limiting the translation. Wang et al. [85] used small RNA and mRNA degradome sequencing to recognize mi RNAs which are high- and low-temperature stress-responsive and targets genes for them in cotton (*G. hirsutum*). Totally, 319 documented miRNAs and 800 unique miRNAs were recognized, and 168 miRNAs were expressed differentially among different temperature treatments. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes revealed that commonly the miRNAs were from genes, which contribute in oxidation–reduction reaction, response to hormone stimulus, plant–pathogen interaction, photosynthesis, and plant hormone signal transduction pathways [85].

Utilization of molecular tools and genetic engineering in breeding for heat tolerance can minimize the complications of polygenic nature of the traits. Zhang et al. [134] conducted a comparison of expression of certain heat-stress responsive genes between heat-sensitive (ST213 and ST4288) and heat-tolerant (VH260 and MNH456) genotypes of cotton in *G. hirsutum*. Orthologs of particular Arabidopsis genes involved in heat-stress response including three heat shock proteins, two heat-stress transcription factors, and the general stress response genes: calcium dependent stress responder, ANNAT8 and ascorbate peroxidase were studied in cotton. Real time qPCR analysis after heat stress treatment revealed that all genes, excluding the heat-shock protein GHSP26, were entirely induced in the heat-tolerant lines of the genotype VH260 as compared to MNH456. Resilient tolerance to heat stress in VH260 can be attributed to prompt sensing of heat stress and timely induction of several mechanisms functioning in coordination to secure the plants against oxidative stress, protein denaturation and membrane damage leading towards decreasing yield losses and improved boll maintenance during heat stress [134].

5.4.2. Genes for cold tolerance

Plants show various responses to encountered environmental stresses. Exposure to low temperature causes expression of numerous genes coding for the proteins that enhance low temperature tolerance via ABA-dependent and ABA-independent pathways [135]. C/DRE, which is a cis acting element shows response to low temperature separately from action of ABA [127].

Study of Phytochromes and aspects involved in their signal transduction are important due to their involvement in plant development and in numerous genetic/biochemical pathways like in plant flowering and architecture, cotton fiber quality, yield potential and productivity,

regulation of nitrate reductase, in fungal disease resistance, salt tolerance, in cold/freezing and drought tolerance [126]. Kim et al. [127] investigated the involvement of phytochromes in facilitating light signaling associated with cold treatment as a photoreceptor for activation of gene expression in response to cold through C/DRE in *A. thaliana*. They found phytochrome B as key photoreceptor controlling the initiation of cold-stress signaling in light response.

Aquaporins are a class of proteins which were reported to play critical roles in plant abiotic stress tolerance. In cotton, a tonoplast intrinsic protein [6], GhTIP1, was reported to enhance the cold tolerance under freezing conditions [136].

Kargiotidou et al. [137] identified and characterized two Phospholipase Da (PLDa) genes from cultivated tetraploid cotton (*G. hirsutum*). Three exons and two introns were observed in genes. A 98.6% homology was observed in both GrPLDa and GaPLDa with their ORFs encoding a polypeptide of 807 amino acids with an expected molecular mass of 91.6 kDa showing an 81–82% homology with PLDa1 and PLDa2 of *A. thaliana*. At the 5′ end a potential alternative splicing incidence was noticed that did not produce alternative ORFs yet. Genes were induced at cold stress (10°C or less) treatment which was declined to control conditions (growth temperature 25 or 22°C) if plants were adapted at 17°C prior to applying cold treatment. Isoforms were differentially expressed when acclimatized to cold and when under cold stress, light was involved in regulation in expression which was attributed to the products of lipid hydrolysis by the endogenous PLDa changing lipid species and a deviation in levels of the signaling molecule phosphatidic acid (PA) after acclimation or cold stress [137].

The transcription factors C-repeat binding factors/dehydration-responsive element binding proteins (CBFs/DREBs) are involved in controlling the expression of many stress-inducible genes. After screening the cDNA library a cDNA clone, named GhDREB1, was identified from cotton (*G. hirsutum*). Results of northern blot analysis revealed that low temperature and salt stress were causing enhanced synthesis of mRNA of GhDREB1 while effect of abscisic acid (ABA) or drought stress was insignificant in cotton seedlings. Over expression of GhDREB1 in transgenic tobacco (*Nicotiana tabacum*) plants exhibited improved tolerance to low temperature than wild-type plants with enhanced leaf chlorophyll, net photosynthetic rate and proline concentrations. Conversely, the transgenic tobacco plants showed minimum growth and late flowering under normal growth conditions. Interestingly, the transcripts of GhDREB1in seedlings of cotton down regulated by treatment of gibberellic acid (GA3). Promoter analysis of the GhDREB1 gene showed that one low-temperature and four gibberellin-responsive elements were present in promoter. Green fluorescent protein (GFP) signal intensity or β-glucuronidase (GUS) activity caused by the GhDREB1 promoter was remarkably enhanced by low temperature but inhibited by GA 3. These findings proved that GhDREB1 works as a transcription factor and is involved in enhancing cold tolerance also affecting growth and development of plant via GA3 [138]. Another gene from *Gossypium hirsutum*, *GhAGP31*, expressing mainly in roots was shown to play important role in tolerance to cold stress during early seedling development [139].

5.5. Utilization of wild species in breeding programs to enhance traits

Homogeneity at genomic level in cotton germplasm is one of the main cause of halted or dropped cotton production around the world rendering cotton crop prone to biotic and abiotic

stresses. Certain wild species possess unique traits including resistance to drought and heat. Valuable traits can be introduced in cultivated cotton varieties using hybridization of various species like, *G. arboreum*, *G. herbaceum*, *G. gossypioides*, and *G. laxum* with *G. hirsutum* and/or *G. barbadense*, afterwards using culture media to raise embryos which may ease in breaking cytogenetic hurdles. This method can be utilized to broaden the genetic base and also for transferring genes involved in traits that are absent in the cultivated species [70]. Enormous variation is present among the cotton germplasm for cold tolerance. Bolek [140] screened 106 cotton genotypes including *G. hirsutum*, *G. barbadense* and *G. herbaceum* for cold tolerance regarding germination efficiency, and found that *G. barbadense* had higher cold tolerance than other species. Thus these genotypes can be utilized in distant breeding program to enhance cold tolerance of our cultivated cotton cultivars.

Cotton genome sequencing accompanied with novel experimentations like nested association mapping based studies, and utility of TILLING populations can be more prolific for cotton breeding. Information generated in genomes, which are well studied, can be interpreted in less explored genomes with the help of comparative mapping. Improved knowledge about evolutionary relations of cotton and Arabidopsis have facilitated deciphering respective gene localization in both genomes which can lead to isolation of full length genes in cotton after getting knowledge about their function from Arabidopsis. This information will also facilitate improvement of translational genomic tools after sequencing of cotton genomes and also in elaborating biological pathways. The sequenced cotton genomes *G. arboreum* [141], *G. raimondii* [149], *G. hirsutum* [142] and *G. barbadense* [143] can be explored for trivial variations at nucleotide level that could be involved in controlling specific traits of cotton. The problem of narrow genetic base of cotton, which is the result of rigorous selection for desired traits, can be solved by getting alleles from wild ancestors [144]. Tetraploid cotton of exotic areas are comparatively heat and drought tolerant. For example, the arid, rocky and clay coastal plains of Hawaii are homeland of GT (http://kalama.doe.hawaii.edu/hern95/pt009/Ann/mccnativeplants.html). Interspecific crossing between GT and GH could produce limited water stress tolerant progenies [145]. A significant variation in WUE, dry matter accumulation, root length and heat tolerance was found amid exotic GH lines [146]. Although development of interspecific hybrids and their utility in breeding programs is very challenging [147].

5.6. Utilization of modern techniques to improve cotton genome against high or low temperature stresses

Whole genome sequencing has revolutionized the genome science. Genotyping-by-sequencing (GBS) is also an alternative lower cost method to identify and score multitude of genome-wide single nucleotide polymorphism (SNP) markers through multiple individuals from miscellaneous populations. Moreover, remote sensing and proximal sensing technologies are promising for the speedy, non-invasive measurement of canopy traits related to the response of cotton to drought and heat stresses in the field. Satellite and aircraft based systems are very informative in context to spectral reflectance and canopy thermal emittance data to be utilized in observing the growth patterns and physiological responses of cotton cultivars grown in field conditions. Hand-held, noncontact sensors when passing through field plots on foot can perform proximal sensing in cotton [23]. Expansion of breeding programmes at genetic level

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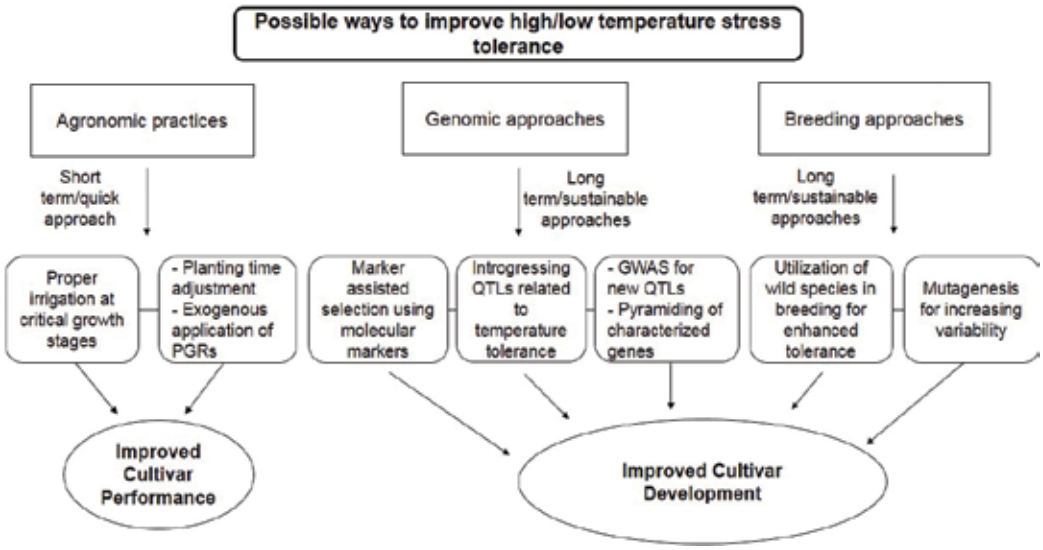

Figure 3. Possible ways to improve high and/or low temperature stress tolerance in cotton.

is significant. Utility of conventional QTL mapping as well as genome-wide association mapping is required to enhance tolerance to temperature extremes. In addition, global expression profiling techniques together with RNA-Seq and miscellaneous omics platforms can be helpful in understanding the fundamental mechanism and selection of the candidate gene (s) for downstream utility. These new techniques are immensely helpful in plant breeding [148]. A schematic diagram for various short and long term possibilities to improve high and low temperature stress tolerance in cotton are shown in **Figure 3**.

6. Conclusion

Changing climate has been creating extreme temperature in many countries around the globe. Temperature stress, more importantly, high temperature has multiple effects on cotton growth and production affecting its yield negatively. In this chapter, we concluded that adopting multiple strategies rather than relying on a single approach is imperative to minimize the losses to cotton production. Breeding temperature tolerant cultivars would be a sustainable and cheapest approach to get good produce under extreme temperature situation. For this, selection of good parents, wild relatives and identification of target genes or markers are of prime importance to start a breeding program.

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Conflict of interest

We confirm that there are no conflicts of interest.

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Chapter 5

Genetic Mapping in Cotton

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Abstract

The genus *Gossypium* provides natural fiber for textile industry worldwide. Genetic improvement in cotton for traits of interest is not up to mark due to scarcity of adequate information about fiber production and quality. Use of DNA markers for overcoming the issues of selection associated with complex traits is the ultimate choice which may lead to initiate breeding by design. Numerous marker-trait associations have been identified for economical traits using linkage analysis in cotton. Currently there is need for developing high-density genetic maps using next-generation sequencing approaches together with genome-wide association studies (GWAS). Efforts have been started in this direction and several QTLs including fiber quality, yield traits, plant architecture, stomatal conductance and verticillium wilt resistance were identified. This chapter narrates genetic diversity, QTL mapping, association mapping and QTLs related to fiber quality traits. The incorporation of various genomic approaches and previously described marker strategies will pave the way for increase in fiber production.

Keywords: cotton, fiber, association mapping, QTLs

1. Introduction

Cotton (*Gossypium spp.*) belongs to the genus *Gossypium*, family Malvaceae and order Malvales, and is known as an ultimate source to produce natural fiber. All over the world, cotton seed is one of the important sources of edible oil. Cotton provides raw material for millions of consumers as well as for industrial products throughout the world. Total impact of cotton in the textile industry continues to excel its importance (presently exceeding 500 billion US$) [1].

Geographically cotton is distributed at 36° South latitude and 46° North latitude in tropical and subtropical regions of the world. The total share of northern hemisphere in global cotton production is 90%. Planting time in the northern hemisphere is the time of harvesting in the southern hemisphere [2].

Cotton is a warm climate crop (cultivated in nearly 100 countries), and is largely grown in Asia, America and Africa. Major emphasis of cotton breeding programs is to improve its lint yield and its quality. It has been thoroughly studied that yield, yield components and fiber quality characters are governed by a number of genes and these are inversely related to each other. Fiber quality and other economic characters have not been refined with conventional breeding strategies as these are adversely influenced by the ecological conditions.

Molecular markers produce variability at genotypic basis and speed up breeding process. Genetic maps are constructed from DNA-based markers information and quantitative trait loci (QTLs) related to trait of interest have been identified. The availability of reference genome of upland cotton (*G. hirsutum* L.). Egyptian cotton (*G. barbadense* L.) and draft genome of *G. arboreum* L., *G. herbaceum* L. [3] and *G. raimondii* [4] has revolutionized the 'omics' studies. The advent of next-generation sequencing with high-throughput sequencing has allowed genotyping at single nucleotide level which are contributing a lot. High uniformity, strength, extensibility, and other fiber quality traits are need of the day worldwide [5, 6]. Fiber development in cotton is a complicated process-comprised of fiber initiation, elongation (primary wall synthesis), wall thickening (secondary wall synthesis) and desiccation (maturation) [7, 8]. Lint and fuzz cover the seed coat of cotton lint serves as a natural textile fiber while fuzz remains on seed coat after ginning.

2. Evolution of genome size in cotton

Polyploidy is a vital evolutionary process in angiosperms; one of the vital factors in creating new plant species [9–11]. Around 70% of the existing angiosperms are polyploids, which include many world-leading crops such as cotton, wheat, potatoes, canola, sugarcane, oats, peanut, tobacco, rose, alfalfa, coffee and banana [11, 12]. Nonetheless, genomic studies in polyploids are lagged behind than diploid species due to their polyploidy nature. It is highly tiresome to create a reference genome in tetraploid cotton owing to involvement of different species. However, advancements in genomic studies like quantitative trait locus (QTL) mapping, association mapping, nested association mapping, cloning, genome sequencing, functional and comparative genomics have laid down the foundation to study such complex organisms for the evolution of highly saturated genetic maps to ascertain the genomic evolution.

In polyploids, after the occurrence of whole genome duplication (WGD), intra and inter-chromosomal rearrangement processes have reallocated both large and small segments across the genome over the evolutionary span. Genome decomposition has given rise to a set of duplicated DNA segments which are dispersed among the chromosomes, with all the duplicate pairs exhibiting a similar degree of sequence discrepancy [11, 12].

The genus *Gossypium* has a long taxonomic and evolutionary history [13]. *Gossypium* is comprised of 52 species, including 46 diploids (2n = 2x = 26) and 5 allotetraploids including one

purporated tetraplod species (2n = 4x = 52) [3]. Out of these, only four species are domesticated. In total, two species are old world diploids (*G. arboreum* L. and *G. herbaceum* L.) and two species are new world allopolyploids (*G. hirsutum* L. and *G. barbadense* L.) which are consisted of a (~1700 Mb) A_t and (~900 Mb) D_t genome. In total four domesticated species contribute toward the production of natural fiber worldwide [14]. *G. hirsutum* L. also known as upland cotton dominates the world's cotton production i.e., > 95%. *G. barbadense* L. known as extra-long staple or sea-island cotton is grown on 2–3% area in the world, but has lower yield/hectare compared to the *G. hirsutum* L. Cultivation of the diploid cotton like *G. arboreum* L. and *G. herbaceum* L. is restricted to a few countries, such as Pakistan and India. Diploid species (2n = 26) are grouped into eight genomic groups (A–G, and K), based on similarities of chromosome pairing [15]. Eight genomes are divided into three different clades as shown in **Figure 1** as A, B, E, and F; D; C, G, K genomes [16], are found naturally in Africa and Asia. D genome clade is indigenous to the Americas and is found in Australia (**Figure 1**).

Tetraploid species evolved ~1–2 million years ago (MYA) as a result of hybridization between "A" and "D" genome species [16], diverged each other from a common ancestor about 4–11 (MYA) [6, 17]. Both "A" & "D" genomes have maintained some level of sequence similarity, resulting in a high transferability of markers among the *Gossypium* species [18, 19]. F_{1s} of mostly cultivated cotton species (*G. hirsutum* L. and *G. barbadense* L.) can further be used in making the crosses with wild tetraploid species (*G. darwinii* G. Watt, *G. mustelinum* Miers ex G. Watt, *G. tomentosum* Nutt. ex Seem.) which produce normal hybrids and some productive off springs [20].

During the last couple of years, major emphasis of genomic research is on comparative analyses of closely related, homoeologous stretch of genomic sequence in plants i.e., maize, rice, and sorghum [21–23]. Cultivated upland cotton has a history of genetic bottlenecks in evolution that have significantly reduced the extent of genetic diversity of the cultivated cotton species, which compelled geneticists to use populations developed by hybridizing two different species for identifying high number of polymorphisms.

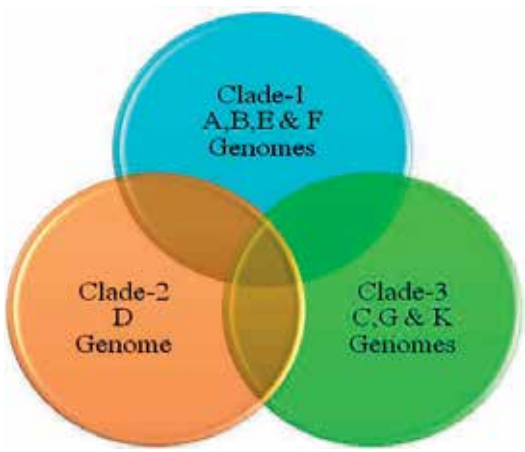

Figure 1. Clades formation in *Gossypium*.

2.1. Genetic diversity in cotton

Cotton has a narrow genetic base which is the main hindrance in sustaining cotton productivity worldwide. Limited genetic diversity and low efficiency of traditional selection methods were the major factors to slow down the process of cultivar improvement from the last three decades [24–26]. One of the major reasons for limited variability of cotton cultivars is the use of adapted cotton germplasm in breeding program. The cotton breeders avoid using the wild genetic resources because of the problem of linkage drags of unwanted characters. The other reason is the lack of innovative tools to mobilize the useful genetic variations from diverse exotic cotton species of *Gossypium* genus into the breeding cultivars. All these factors together led to the genetic bottleneck in evolution [27]. Understanding about the extent of genetic diversity and relationships among breeding materials could pave the way for precise parental selection and germplasm organization for cotton improvement breeding programs [28–34]. Percent Disagreement Values (PDVs) distance matrix, tree clustering diagram and neighbor-joining stars are different statistical techniques to determine the extent of genetic diversity. Polymorphism Information Content (PIC) is another statistical technique that can be deployed to evaluate the polymorphism acquired through different techniques, primers or markers [35–38].

2.2. Genomic studies in cotton

The discoveries made through exploring the genome would set a firm foundation for initiating breeding by design for improvement programs in cotton. Over the last two decades, multiple genomic tools have been utilized for exploring the cotton genome. Different types of DNA markers such as restriction fragment length polymorphism (RFLP) [39, 40], randomly amplified polymorphic DNA (RAPD) [41–48], amplified fragment length polymorphism (AFLP) [27, 49], simple sequence repeat (SSR) or microsatellites [37, 50, 51], single nucleotide polymorphism (SNPs) [52–55], physical maps, genetic maps, mapped genes and QTLs, microarrays, gene expression profiling, BAC and BIBAC libraries, QTL fine mapping, resistance gene analogs (RGA), genome sequencing, non-fiber and non-ovule EST development, gene expression profiling, and association studies for various traits have been extensively used for understanding the cotton genome. Finally, the genome sequence information of *G. hirsutum* L. and its progenitor species will considerably expedite the cotton genomic research toward identifying new genes conferring various traits of interest, and would also help in identifying DNA markers linked with traits which can be used in MAS.

2.3. Mapping population

The group of individuals used for the determining variation on genetic basis, phylogenetic analysis, development of genetic map, assigning of loci to the trait of interest is known as mapping population; which are of vital importance for mapping. Mapping populations are obtained using two contrasting parents for the desired trait. In self-pollinated crops; usually mapping populations include F_2 [56, 57]; $F_{2:3}$ [58, 59], recombinant inbred lines (RILs) [60, 61], backcross (BC) [62, 63], Backcross inbred lines (BILs) [64], near isogenic lines [65, 66], double-haploids [67, 68], chromosome substitution lines (CSILs) [69, 70]. F_2, BC, RILs and double-haploids have been highly used for linkage mapping studies in cotton.

The easiest developed populations include F_2 and BC as less duration is required. F_2 population has more cons for detecting QTLs with additive effects and can also be utilized for assessing the dominance pattern. A number of QTLs for cotton has been found using this population [56, 71–73]. Backcross population produce false results if dominant factors are allowed as additive and dominance are overlapped. Nonetheless, these two populations have demerits including (i) owing to less meiosis some markers which are present at a distance from QTL are also counted; (ii) non-allelic interaction cannot be analyzed; (iii) F_2 and BC have got more heterozygosity and also temporary as cannot be repeated at different locations. In-contrast RILs have gone through number of selfings and are highly homozygous [63]. Moreover, RILs are populations which produce finely saturated genetic maps as recombination frequency is high. RILs have been used in cotton for identifying traits related to agronomic and fiber [5, 53, 74]. Doubled-haploids are the best populations for improving any trait as these are the ones having 100% purity. These can be obtained in less duration compared to RILs and BILs but needs to be developed in a fully sterile environment with high skill [75]. By using these, it's convenient to reduce the variety development duration and analyze genetic behavior. BILs, RILs and double-haploids are the permanent populations and the inferences can be analyzed in detail of QTLs after phenotypical screening, genotyping and genetic map construction.

2.4. Association mapping

During the last decade, interest of plant geneticists is increasing to use the nonrandom associations of loci in haplotypes, a powerful high-resolution mapping tool for studying the complex quantitative traits as compared to the conventional linkage mapping. Association between chromosomal fragments and phenotype can be determined through exploiting genotypic data. Genotypic and phenotypic data are collected from a population with unknown relatedness followed by the estimation of marker-trait association in the experimental population. Association mapping is an open system model that helps in developing high-resolution maps while in linkage mapping, fine mapping is required to reach near the loci [76], but understanding about the time and place of recombination in the genome is very tricky. Single-marker analysis, interval mapping, multiple interval mapping, and Bayesian interval mapping, have been widely used in conventional linkage mapping studies. Association mapping is an influential approach to map genes for QTLs using genomic tools together with robust statistical methods. Association mapping is an imperative way to investigate the genetic structure of QTLs which can lay down a foundation to study the different traits like insect resistance, disease resistance, earliness, fiber quality etc. [77]. Zhu et al. [78] reviewed status and prospects of association mapping in comparison to linkage analysis. However, recent advances in association of DNA markers with the fiber quality traits paves the way to understand the mechanism of cotton fiber development.

Linkage disequilibrium mapping (LD) commonly named as association mapping is a method to detect and locate QTLs based on marker-trait association study and it anticipates a relatively new method to dissect the complex traits. Methods for linkage disequilibrium were initially developed for undertaking human genetic studies [79, 80]. These methods have been successfully translated on crop plants for exploring the linkage disequilibrium (LD). Association mapping offers a uniquely high-resolution mapping strategy based upon historical

recombination events at population scale which can empower mapping at gene level in less studied organisms where conventional QTL mapping would not be practical [81].

There are several ways for the determination of LD [82] but the most popular statistic parameter for the calculation of marker-trait association is "r^2". Theoretically Pearson's correlation coefficient narrates the polymorphism of allele at one locus to other allele at another while "r^2" is known as "coefficient of determination" being the squared value of Pearson's coefficient. As a whole "r^2" elaborates the magnitude of individual variance independent variable with the dependent variable when linear regression is accomplished.

LD is described by another common statistic parameter termed as "Lewontin's D". If two loci are segregated randomly then "D" measures the disequilibrium as the distinction among coupling and repulsion gametes frequencies [83]. D is used for calculating D' for determination of association among loci using the formula:

$$D = 2\,P_{AB} - P_A \times P_B \tag{1}$$

P_{AB} is the observed extent of a set of closely linked alleles of two loci inherited to offspring with allele A in the 1st locus and B in the 2nd while PA is allele A frequency in 1st site and PB is allele B frequency on 2nd. Owing to base on allelic magnitude the D calculated value is not a precise approach for determining power and distinction of nonrandom association. D' was developed by Lewontin [83] for determining the LD which is less related to allelic magnitude. Varshney and Tuberosa [81] revealed that LD variance values were often high but D' had minimum variance nonetheless the individuals were evaluated from populations under equilibrium. He also pointed out that population size had significant impact upon association as D' can produce problematic outcomes for the studies.

Ersoz et al. [84] devised other approaches based on kinship which deals with the determination of probability of independence among two loci through individual spreading instead of using LD statistics summary. These statistical tools are also known as model-based LD methods which allow determination of population recombination measure from sequence information in an unbiased equilibrium model [85–87]. Besides these models there are other ways which are model-based using diverse population structures for the calculation of LD for differentiation among different individuals origin [88].

The applications of association mapping are receiving major attention for genomic studies of quantitative traits in all major crops. However, association mapping achieved for crop improvement is not comparable to that in human genomics [89]. Over the decades, many QTLs have been identified using bi-parental populations for yield, yield components and other traits of interest [90, 91]. However, only few were successfully used in plant improvement programs. The recent advancements in genomic science has provided the opportunity of identifying more QTLs through various approaches including GWAS. Similarly, low cost genotyping methods also complemented the aforementioned in identifying more QTLs which can be used in breeding programs. A genome-wide association study (GWAS) was conducted for yield components and fiber quality traits on a diversity panel of 103 cotton accessions. They identified 17 SNP associations for fiber length and 50 for micronaire value [191]. In another report, GWAS

was conducted on 318 genotypes. They found that 54.8% of the GWAS detected alleles were transferred from three founder parents; Deltapine15, Stoneville 2B and Uganda Mian [192].

DNA markers linked to QTLs contributing toward traits of agronomic importance are invaluable resources for cotton (*Gossypium* sp.) improvement. In spite of the existence of potential diversity in the *Gossypium* genus, it is mainly underutilized due to barriers of photoperiodism and stringency of advanced technologies to deal with these challenges. Linkage disequilibrium (LD) mapping is a powerful tool for dissecting genetic diversity. Abdurakhmonov et al. [92] used association mapping in 208 exotic *G. hirsutum* L. accessions, containing 208 landrace accessions and 77 photoperiodic accessions. A significant genetic diversity within exotic germplasm stock was found. About 11–12% of SSR loci showed significant LD. Estimates of LD declined at significant threshold ($r^2 = 0.1$) found in the range of 10 cM genetic distance in landraces and 30 cM in varieties. LD calculated at $r^2 = 0.2$ was estimated on an average 6–8 cM in cotton varieties and ~1–2 cM in land races, providing evidence for potential associations for important traits. A significant relatedness and population structure was found in the germplasm. Mixed linear model (MLM) detected between 13 and 6% of SSRs associated with major fiber quality parameters in cotton. The study demonstrated the potential application of association mapping in cotton to exploit new sources of genetic variation.

Utility of the diploid Asiatic cotton species in breeding programs depend upon the understanding of the ancestry and genetic relatedness. A collection of 56 *G. arboreum* L. accessions collected from nine different zones of Asia, Africa and Europe were assessed for eight fiber quality parameters (strength, lint color, lint percentage, micronaire, elongation, maturity, 50% span length and 2.5% span length) and genotyped with 98 microsatellites. Majority of the SSRs were found polymorphic. The analysis of population structure identified six major clusters for accessions representing distinct geographic regions. Marker-trait association estimates were assessed by general linear model method. This study illustrated the potential of association mapping in diploid cotton, because a modest number of SSRs, phenotypic data and strong pioneering statistical interpretation, identified interesting associations [93].

The use of marker-assisted breeding (MAB) in cotton improvement is limited, as compared to the other commercial crops due to its narrow genetic base and limited polymorphisms. This scenario urges a need for tagging, characterization and utilization of naturally existing polymorphisms in *Gossypium* germplasm collections. Estimates of genetic diversity, population structure, LD magnitude and association mapping were explored for cotton fiber quality trait in a set of 335 *G. hirsutum* L. germplasm cultivated under two distinct environments by surveying 202 SSRs. Genome-wide LD at $r^2 \geq 0.1$, extended up to 25 cM in tested cotton accessions. However, at a threshold of $r^2 \geq 0.2$, genome-wide was reduced to ~566 cM, highlighting the potential application of association mapping studies in cotton. Preliminary findings suggested inbreeding, linkage, selection, genetic drift and population stratification as the key LD-generating players in cotton. Using a kinship and unified MLM on an average ~20 SSR markers were observed to be associated with major fiber quality traits in two environments. These significant associations were further confirmed for permutation based multiple testing and population structure by applying linear model and structured association test. The identified association provided a strong evidence for the use of association mapping studies in cotton germplasm resources [94].

In another report, association studies were undertaken to identify SSR markers linked with fiber traits in the exotic germplasm population derived from multiple crosses among tetraploid species of *Gossypium*. After 12 generations of continuous selfing, a total of 260 lines were selected for evaluation of fiber-related traits in three environments from species polycross (SP) population. A total of 314 polymorphic fragments were amplified by surveying with 86 SSRs. The SSRs showing 6% allele frequency were evaluated for associations. A total of 59 markers have substantial ($P < 0.05$, 0.01, or 0.001) association with six fiber traits. Structure analysis grouped the population in six groups with allelic frequency ranged from 0.11 to 0.27. The correction for population structure and kinship analysis identified 39 out of 59, significant marker-trait associations. Population sub-structure was highly significant for boll weight. The results clearly indicated that marker-trait associations have a promising potential in determining the genetics underlying interrelationships among fiber traits [95].

The discovery of valuable alleles for fiber quality traits and also the novel germplasm exhibiting high fiber quality features are important for accelerating the breeding progress for improving the lint quality. An association mapping study was conducted for fiber quality traits using 99 *G. hirsutum* L. accessions with diverse origins. A total of 97 polymorphic microsatellite marker were used which detected 107 significant marker-trait associations for three fiber quality traits under three diverse environments. A total of 70 marker-trait significant associations were detected in two to three environments while 37 identified in only one environment. Out of the 70 marker-trait associations, 52% were found similar with earlier reports, indicating the stability of these loci for the target traits. Further, a large number of elite alleles conferring two or three traits were also detected. These results pointed out the potential of using germplasm for mining elite alleles and their use in breeding for improving the lint quality [71].

Knowledge about population structure and linkage disequilibrium in association mapping studies can help in minimizing the appearance of false positive associations. Association mapping of verticillium wilt resistance in cotton was reported in the panel of 158 cotton genotypes. The studied germplasm was genotyped with 212 markers covering the whole genome and phenotyped with disease nursery and screening method in green house. In total 480 alleles were identified, ranged 2–4 alleles/locus. A total of two major groups and seven subgroups were identified through model-based analysis. The LD level of the linked markers was considerably higher than the unlinked markers, indicating that physical linkage heavily affected LD in this panel and LD level increased when the studied germplasm was divided into groups and subgroups. In total, 42 marker loci were associated with verticillium wilt resistance, which were mapped on 15 chromosomes. In total, 10 out of 42 marker loci were found to be constant with already known QTLs while 32 were new marker loci. This study paved the way for marker-assisted selection of verticillium wilt resistance in cotton [96].

Baytar et al. [97] used SSRs in a germplasm collection consisting of 108 genotypes for association analysis and analyzing genetic diversity, population stratification and linkage disequilibrium in upland cotton. 967 alleles were used for population construction and differentiated into 4-subgroups. Linkage disequilibrium showed the decay in 20–30 cM ($r^2 \leq 0.5$) and association was observed via general linear model and mixed linear model for verticillium disease resistance. As a whole 26 markers were observed associated with this disease on

14-chromosomes at $P \leq 0.05$ while it was found that 8 of total markers were highly significant $P \leq 0.01$. Phenotypically variation fluctuated in each marker from 3.2 to 8.2%. They assumed that the identified markers being in accordance to earlier studies may be a good source for devising any breeding strategy.

The genetic pattern of sympodial branch number, length, node of first fruiting branch and some other characters of branches was observed in an association panel of 39 genotypes and 178 F_{1s} under separate ecological conditions for developing ideogram, photosynthesis and yield [98]. 20 QTLs were found for these traits with MLM in association analysis which revealed that these traits had additive, dominance, epistatic and environment effects and phenotypical variation showed these traits are highly influenced by genetic factors.

2.5. Fiber quality traits

World cotton consumption increased 2.9% from 2012 to 2013 (106.4 million bales) to 2013–2014 (109.5 million bales) [99]. Cotton being the prime fiber crop of the world [100], and cash crop of Pakistan which have a significant contribution as foreign exchange in the economy of country [101].

Globally, the demand for cotton products is projected to rise 102% from 2000 to 2030. This is likely to occur in a global environment where arable land is squeezing, water supplies are decreasing, and the impact of worldwide climate change on cotton production is uncertain. Current rate of genetic gain for lint yield under normal plant densities range from 7.1 to 8.7 kg ha^{-1} year [102]. Most of the genetic gain has been achieved through deploying conventional breeding tools and recently the biotechnological tools [103]. Conventional breeding alone cannot achieve the genetic gain without supplementing it with modern genomic tools. Fortunately, cotton genomic research has gained momentum after the introduction of GM cotton [104]. The other approaches including marker-assisted selection (MAS) can accelerate the breeding progress. Genetic variations for fiber quality traits among *G. hirsutum* L. cotton substantially limit its quality improvement [45, 105]. Breeding for better quality lint is a primary objective of most cotton breeding programs worldwide. Conventional breeding has played a key role for improving yield and fiber quality of upland cotton. The invention and advancement of molecular markers surely make it accessible for plant breeders to even more rapidly and precisely improve crop economic and agronomic traits [106]. Cotton fiber originates from the seed protodermal cells, being the renewable of textile materials and major alternative to man-made fibers.

Cotton genotypes significantly differ for fiber quality traits [107] and lint percentage [108]. Different types of the model systems can be used to discover the new genes controlling the cotton fiber. Cotton seed hair development has a strong resemblance with Arabidopsis leaf trichome development [109]. The data generated from different studies using cotton fiber-related genes supported this study, thus confirmed the close relationship between cotton seed fibers and Arabidopsis trichomes [110–112].

Researchers working on cotton fiber development demonstrated that, there is a significant impact of high-density genetic map of cotton anchored with fiber-related genes which may expedite the MAS to improve fiber quality traits. Keeping this object in mind, a genetic map

was constructed by deploying simple and complex sequence repeat markers on 183 recombinant inbred lines (RILs) derived from the interspecific cross TM1 (*G. hirsutum* L.)/Pima 3–79 (*G. barbadense* L.). The newly developed genetic map was comprised of 193 loci including 121 new fiber loci not previously reported. These new reported fiber loci were mapped on chromosome no. 19 and 11 LG extending 1277 cM, contributing approximately 27% of the total genome coverage. Preliminary QTL analysis studies suggested that genes for fiber-related traits were present on chromosome no. 2, 3, 15 and 18. These newly synthesized PCR-based SSRs derived from cotton fiber ESTs will open new doors for the development of a high-resolution integrated genetic map of cotton for structural and functional study of the genes that augment fiber quality [74].

Jamshed et al. [113] screened 28,861 SSRs to identify polymorphism among parents 0–153 and sGK9708 and used 851 polymorphic SSRs in a RIL population containing 186 individuals for determining genomic regions connected to fiber quality. The genetic map spanned to 4110 cM with 5.2 cM distance between makers and as whole constituted about 93.2% *G. hirsutum* L. genome. As a whole they found 165 QTLs related to fiber and 90 of them were declared as common QTLs which will be a good source for cotton.

In another study, researchers reported that the mapping of genes involved in cotton fiber development will expedite the cloning and manipulation of these genes. In this study, already known seven fiber mutants were mapped, four dominant (Li_1, Li_2, N_1 and *Fbl*) and three recessives (n_2, *sma-4*(h_a), and *sma-4*(*fz*)) in six F_2 populations spanning 124 or more plants each. Map position of the mutants were harmonious with previously assigned chromosomes except n_2, which was mapped on the homoeolog of the chromosome already reported. Three mutations (N_1, *Fbl*, n_2) having primary effects on fuzz fibers were mapped near QTLs that affected fiber lint production in the same populations that may be due to pleiotropic effects on both fiber types. However, only one mutant Li_1 mapped within the likelihood interval for 191 already reported lint fiber QTLs discovered in non-mutant crosses, suggesting that these mutations may occur in genes that played early roles in the evolution of cotton fiber and for which new allelic variants are quickly eliminated from improved germplasm. Studying the genome comparison of cotton and Arabidopsis opens new avenues to accelerate the genetic dissection of cotton fiber development [114].

Genetics of the fiber traits was determined in a cross of 5 × 5 complete diallel system. This study reported additive gene action, demonstrating that fiber quality of a certain cotton genotype is a sequence of different fiber quality traits. However, the most important traits are fiber length, strength, fineness and uniformity index [115].

Bhatti [55] observed association for fiber quality traits in a global germplasm collection of upland cotton using SNPs. 32 QTLs found connected to different fiber traits such as fiber length, fiber strength, uniformity index, micronaire, maturity, fiber strength and fiber elongation.

2.5.1. Ginning out turn percentage

Lint is the lifeline of the textile industry, serves as a backbone in earning foreign exchange and thus adds up in country exchange reserves. Ginning out turn (GOT) percentage has a key role for more lint production. GOT percentage is a useful index for the performance of

a genotype and it can be defined as the percentage of lint obtained from a sample of seed cotton. Genotypes-cultivars having high GOT are preferred, because of high lint potential. Approximately, 1% increase in GOT would bring about 3% increase in seed cotton yield. In order to meet the demand of textile industry, the breeders ought to breed for high lint producing genotypes-cultivars.

Genetic make-up of a cotton plant contributes more toward lint than that of the macronutrients, phosphorus and nitrogen level [116, 117]. Motes percentage and GOT are highly affected by genotypes-cultivars and location [118]. Effect of sowing time on GOT has also been reported in multiple studies [119, 120]. Negative correlation between staple length and GOT has been observed [121] while GOT is directly associated with seed cotton yield [122]. Non-additive gene action has been reported for GOT, irrespective of its high heritability estimates [123]. Applications of farm yard manure can improve the fiber yield by improving GOT [124].

A total of 25 QTLs conferring lint production were reported over the entire genome but none of the chromosome contained more than three QTLs [91]. Qin et al. [125] reported 17 associated markers with lint percentage.

12 QTLs connected to lint percentage were observed at whole genome level in upland cotton [126]. They also assumed that QTL Gh_A02G1268 was also found in fiber development and these QTLs can be used for fiber improvement at whole genome level.

Zhang et al. [127] developed chromosome introgressed lines using TM-1 and TX-256 and TX-1046 and observed 5 QTLs related to ginning out turn and concluded 1 stable QTL which was observed in multiple environments.

Iqbal and Rahman [128] screened germplasm collection of 185 genotypes with 95 polymorphic SSRs for three years and at different locations for ascertaining lint percentage. They found IR-NIBGE-3701 with the maximum GOT percentage of 43.63%. The population pattern was observed using STRUCTURE, unweighted pair group method with arithmetic mean and principal component analysis and four clusters were developed. Totally 47 genotypes found to have common ancestry and distance among subgroups ranged from 0.058 to 0.130. As a whole 75 marker-trait associations were detected among fiber quality traits; out of which 18 were related to GOT percentage and MGHES-51 found in all traits. They concluded that such QTLs can be utilized in molecular breeding as a tool to observe all quality traits.

2.5.2. Micronaire value

Fiber fineness and maturity are measured in terms of micronaire value because this value is the combination of fiber maturity and fineness [129, 130]. Micronaire value is the measure of air resistance through plugs of cotton, wool, rayon, and glass wool fibers [11, 131, 132]. Optimal range for micronaire value is 3.8–4.5. Lint with high micronaire > 4.5 is considered as of coarse quality that results in less fiber in the yarn cross-section. Ultimately, the course fiber makes relatively weaker yarn. It is one of the reasons that high micronaire cotton is less preferred by the spinners due to reduced fiber bundle strength [133]. Less micronaire value < 3.5 μg/inch of lint is also undesirable as it reflects the immature fiber which may prone to dye uptake problems, breakage and neps formation.

Biotic and abiotic stresses have a major impact on micronaire value. For example, temperature, plant defoliation [133–135], radiation [136, 137] and water stress [138] significantly impact the micronaire value. Thus, understanding the extent that these factors affect the micronaire value is important for undertaking cultural practices to produce cotton fiber with desirable micronaire value.

Various instruments have been developed for the accurate measurement of micronaire value like areal meter [139], shirley fineness maturity tester (FMT), originally developed by Shirely institute, since 1998 sold as the WIRA Electronic Cotton Fineness and Maturity Meter [140, 141] and the Uster Technologies Advanced Fibre Information System (AFIS) providing module for direct measurement of individual fiber diameter and giving the degree of fineness [142, 143]. Now a days, latest instruments Cottonscan and Siromat for the measurement of micronaire are commercially available in one instrument called Cottonscope [144].

Fiber quality assessed in a population developed from contrasting parents and SSRs used for determining associations between economic traits [145]. They found 131 QTLs for fiber quality, verification done in another RIL population and deduced that 77 QTLs were in accordance to earlier findings while 54 are unique and will fasten MAS in cotton.

Said et al. [91] identified 234 QTLs for micronaire value, which were spread over the entire genome of cotton. Most of these micronaire QTLs were on chromosome no. 5, 24 and 25.

Zhang et al. [127] observed QTLs related to fiber length, micronaire and strength using SSR and SNPs in a RIL population consisted of 196 individuals developed from 0 to 153 and sGK9708. They identified 25 QTLs on chromosome 25 and 17 among them were common in minimum two locations. They also detected a specific genomic region for micronaire COT002-CRI-CRI-SNP68652 which will contribute a lot for fiber quality improvement.

2.5.3. Staple length

Staple length is the average length of the longer one half of the fibers. Its improvement through adopting various breeding procedures including the modern genetic tools is the only effective way. It is manifested by high heritability-ranged from 0.52 to 0.90 for fiber bundle strength and 0.46 to 0.79 for staple length [146–149]. Previous studies have shown that staple length is highly under the influence of the genotype [69]. In the present cultivated varieties, significantly low variations for the staple length were observed-thus hampering the future breeding progress because of low genetic diversity available for the trait [25, 150, 151].

A total of 151 staple length QTLs were reported over the entire genome except chromosomes C2 and C22 [91]. In total 12 marker-trait association for staple length were reported in earlier study [125]. Cuming [152] reported four QTLS for staple length in the genetic mapping of F_2 population in the green colored cotton.

There is a dire need to broaden the genetic base of cultivated upland cotton for continuous genetic advancement of seed cotton yield and fiber-related traits through introgressing the alleles from *G. barbadense* L.

Tan et al. [153] used RIL population and screened SSR for developing QTLs related to fiber quality. As a whole 59 QTLs were found related to fiber quality; 15, 10, 9, 10, 15 for fiber length, uniformity, strength, elongation and micronaire respectively. They revealed that these QTLs can be used for developing cultivars in upland cotton.

Association analysis conducted for fiber quality using CottonSNP63K in a germplasm collection of 503 genotypes at genome-wide level [154]. The populations were differentiated into three subgroups on the basis of 11975 SNPs and found that genetic structure is not based on geographic based. They observed 160 QTLs associated with yield and yield components with 324 SNPs.

2.5.4. Fiber bundle strength

Fiber tensile properties include fiber bundle strength and elongation. HVI based tensile properties are user friendly and provide average estimates for thousands of fibers. Single fiber tensile testing is a tedious job and thus not routinely practiced [155] but it has been observed that single fiber testing provides better intrinsic fiber tensile properties [156]. Fiber bundle strength has a major impact in the modern spinning technology rather than staple length and micronaire value [157]. Negative correlation between the fiber bundle strength and cotton lint is a major bottle neck in upland cotton breeding programs [158–160]. It means that increase in fiber bundle strength would not be possible without sacrificing the yield. Good quality fiber that contributes to the production of stronger yarn is highly desirable and has a major impact on highly efficient fabric production [161, 162]. Fibers having optimal micronaire value, long staple length and high fiber bundle strength have much more potential to synchronize with textile processing methods while fibers of short staple length have lesser yarn strength which reduces the efficiency of spinning and ultimately decreases the yarn utility. The textile industry requires yarn of high average strength so that it can help to counter harsh spinning activities [163, 164]. Said et al. [91] reported 132 fiber bundle strength QTLs, which were spread over the entire genome with the exception of chromosome no. 17 which contained none. A total of 12 associations between SSR makers and fiber bundle strength were reported in the association mapping studies of *G. hirsutum* L. collections [125]. The exploration of novel genes in the wild germplasm and their introgression into adaptive cultivars would pave the way for the genetic improvement of seed cotton yield and fiber bundle strength in *G. hirsutum* L.

2.5.5. Uniformity index

The fiber uniformity index (uniformity ratio) is the ratio between upper half mean length (UHML) and mean length expressed as a percentage of the longest length. Uniformity index has a major role in cotton spinning industry. It is highly vulnerable to environmental changes with some special reference to micronaire value [165, 166]. That's why it is highly desirable to improve the fiber-related traits including uniformity index to fulfill the needs of the textile industry because low length uniformity and high short fiber contents are correlated with more manufacturing waste and less spinning efficiency during yarn process.

In multiple studies, the inter-relationship between QTLs associated with three fiber length parameters, average staple length, length uniformity and short fiber contents was reported

[62]. Many researchers reported the QTLs for length uniformity, which are useful to produce the uniform cotton [167]. In total 91 QTLs were reported over the entire genome except for the chromosome no. 11, which contained none of the fiber uniformity index QTL [91]. Cuming [152], identified two fiber uniformity index QTLs in the F_2 intraspecific population of *G. hirsutum* L. by deploying single-marker analysis. Moreover, success made in cotton using genetic mapping has been described in **Table 1**.

Species	Pop. type	Pop. size	Markers number	Markers type	Mapped traits	Ref.
G. hrisutum	Diverse accessions	285	95	SSRs	Fiber quality	[92]
	Diverse accessions	335	202	SSRs	Fiber quality	[94]
	Exotic germplasm	260	86	SSRs	Fiber traits	[95]
G. arboreum	Accessions (9-regions)	56	98	SSRs	Fiber quality	[93]
G. hrisutum	Accessions (global)	1000	100	SSRs	Yield and fiber	[168]
	Wild races and variety accessions	37	23	SSRs	Fiber traits	[169]
Cultivated sp. and wild	Accessions	8193	197	SSRs	Verticlium and fiber	[96]
	Germplasm	323	106	SSR	Drought and salt	[170]
	Accessions	323	651	SSR	Yield traits	[171]
	Cultivars	356	381	SSR	Yield and yield components	[172]
	Cultivars	356	185	SSR	Fusarium wilt	[173]
	Germplasm	75	69	AFLPs	Seed quality	[174]
G. hrisutum	Diverse accessions	99	97	SSRs	Fiber quality	[175]
G. hrisutum	Variety germplasm	109	98	SSRs	Salinity	[121]
G. hrisutum	Elite cotton cultivars	180	58	SSRs	Oil and protein	[176]
G. hrisutum	Elite germplasm	158	212	SSRs	Verticilium resistance	[96]
G. hrisutum	cultivars	134	74	SSR	Salt tolerance	[177]
	Germplasm	123	120	SSRs	Fiber traits	[178]
Upland cotton	Accessions	355	81,675	SNPs	Fiber traits	[126]
	Accessions	355 and 185	81,675	SNPs	Early maturity	[179]
	Inbred lines (native and exotic)	503	179	SSRs	Fiber quality	[180]
	Cotton genotypes	90	95	SSRs	Drought tolerance	[181]
	Accessions	172	331	SSRs	Earliness	[182]
	Germplasm	200	3786	SNPs	Yield and fiber quality	[183]

Species	Pop. type	Pop. size	Markers number	Markers type	Mapped traits	Ref.
	Germplasm	108	177	SSR	Verticilium resistance	[97]
	Cultivars	172	331	SSR	Yield traits	[184]
	Accessions	395	103 and 26,324	SSRs and SNPs	Seed protein, oil and traits	[185]
	Accessions	299	85,630	SNPs	Verticillium resistance	[186]
	Accessions	261	535	ILPs	Salt tolerance	[187]
	Germplasm	185	95	SSR	Lint traits	[128]
	Accessions	305	198	SSR	Fiber quality	[188]
	Landraces and cultivars	318			Lint yield and fiber quality	[189]
	Accessions	503	11,975	SNPs	Agronomic traits	[154]
	Diverse accessions	719	10,511	SNPs	Fiber quality	[190]

AFLP, amplified fragment length polymorphism; SSR, simple sequence repeat; SNP, single nucleotide polymorphism, ILP, intron length polymorphism.

Table 1. Success stories of association mapping studies in cotton.

2.6. Conclusion

As cotton is the most common source of natural fiber all over the globe, there is an urgent need to improve its lint yield and quality through the utilization of diverse germplasm resources and employing high-throughput technologies. The development and efficiency of phenotypic traits can be maximized using DNA-based markers, and mapping of cotton can lay the foundation for future breeding strategies. Family-based genetic mapping has been used for ascertaining desirable traits in cotton for a while, however, it may not be as reliable as once though since some regions connected to the trait of interest may be highly influenced by climatic conditions as well. Therefore, breeders have become more inclined to use variations hidden in the permanent populations such as accessions and landraces in gene pool. The associations between various economical characters discovered using such resources provide genetic mappers with valuable information since there would be no recombination between the character and the marker. Advances in emerging technologies in sequencing would require automation and in order to accomplish an efficient automation, the use of highly potent markers would become crucial. SNPs are the markers of choice for such genomic studies because they can be developed by employing different methods. GBS is one of such methods and it is unique in its perspective, as it can detect reproducibility and genotype of large populations simultaneously. When the recent developments in the field are considered altogether, it appears that the incorporation of various genomic approaches and genotyping will pave the way to increase fiber production and they will be the source of food security at global level.

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Chapter 6

Recent Developments in Fiber Genomics of Tetraploid Cotton Species

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Abstract

Cotton (*Gossypium* spp.) produces naturally soft, single-celled trichomes as fiber on the seed coat supplying the main source of natural raw material for the textile industry. It is economically considered as one of the most leading cash crops in the world and evolutionarily very important as a model system for detailed scientific investigations. Cotton production is going through a big transition stage such as losing the market share in competition with the synthetic fibers, high popularity of *Bt* and herbicide resistance genes in cotton cultivars, and the recent shift of fiber demands to meet the standard fiber quality due to change of textile technologies to produce high superior quality of fibers in the global market. Recently, next-generation sequencing technologies through high-throughput sequencing at greatly reduced costs provided opportunities to sequence the diploid and tetraploid cotton genomes. With the availability of large volume of literatures on molecular mapping, new genomic resources, characterization of cotton genomes, discoveries of many novel genes, regulatory elements including small and microRNAs and new genetic tools such as gene silencing or gene editing technique for genome manipulation, this report attempted to provide the readers a comprehensive review on the recent advances of cotton fiber genomics research.

Keywords: fiber genomics, SSR markers, QTL analysis, genome-wide analysis

1. Introduction

Cotton (*Gossypium* spp.), a natural fiber source for the textile industry [1], has significant economic impact in about 80 countries including USA, China, India, Pakistan, Brazil, Egypt, and Uzbekistan [2]. Cotton seeds are a rich source of vegetable oils, medicinal compounds and byproducts. Cottonseed is also used in livestock feed [3]. The estimated world cotton area and production worldwide are 32–34 million hectares and ~26 million metric tons of fiber [4]. Biologically, cotton fibers are single-celled trichomes [5] that grow from the epidermal cell layer of the ovule in a boll. The allotetraploid cotton genomes contain two subgenomes, A_t and D_t, in its nucleus, which resulted from the ancestral allopolyploidization of progenitor A-genome and D-genome diploids. The presence of the A_t and D_t subgenomes further increases the complexity in understanding the evolution, function, and composition of the allotetraploid cotton. Most of the economically important traits in cotton are controlled by complex quantitative trait loci (QTL) composed of many genes that collectively express the phenotype. Not all fiber quality traits are positively related to lint yield, and many have a negative genetic correlation with important agronomic traits including lint yield [6]. The principle of cotton breeding is mainly to select desirable traits based on market demand, and breeders embrace this objective in their genetic improvement programs. Previously, cotton breeders' primarily emphasized yield and agronomic characteristics, but recent technological changes in the spinning industry necessitated genetic improvements in major cotton fiber quality traits such as length, strength, micronaire (fineness), uniformity, reflectance, elongation, short fiber content, etc. [7]. Accordingly, cotton producers demand superior lines that will produce not only high yield but also improved fiber quality.

Although significant progress has been achieved in developing genetically-engineered insect and herbicide resistant cultivars, which suggested usefulness and efficiency of an integration of molecular and conventional methods for the genetic improvement of cotton, there is currently a great challenge to efficiently and rapidly breed complex traits such as fiber quality. One of the great obstacles in this regard is the narrowness of genetic base of current cultivar germplasm, which is due to following reasons: (1) a "genetic bottleneck" occurring during domestication of cotton [8, 9]; and (2) repeatedly using the similar cotton genotypes for breeding programs as a major parents [10]. Many of these above prompted researchers to bring novel tools and change breeding approaches to have successful cotton improvement programs. With advances in modern genomic technologies, considerable progress has been made in utilizing innovative approaches to achieve progress in cotton genetic research and breeding program. This review emphasized the latest advances in molecular mapping, genome-wide analyses, genome sequence, characterization of fiber genes, and existing genomic approaches for the improvement of fiber traits in allotetraploid cotton species.

2. Molecular markers

DNA markers are "landmarks" in the genome that can be selected because of their proximity to a QTL of interest. The selection of DNA markers linked to a QTL increases the efficiency of breeding, and usually decreases costs and subjective phenotypic selection with minimal backcrossing. Molecular markers represent a site of detectable variation in the genomic DNA, and

they are broadly categorized as: (1) restriction enzyme-based DNA markers (such as RFLP and AFLP), (2) polymerase chain reaction (PCR)-based markers (mostly SSRs) and (3) single nucleotide polymorphism (SNP)-based markers.

The first RFLP map in an interspecific cotton population (*G. hirsutum* × *G. barbadense*) with 705 RFLP loci identified 41 linkage groups that covered 4675 cM locations mapped on 11 pairs of homoeologous chromosomes [11]. An RFLP map with 63 fiber QTLs linked to the A-subgenome (chromosome 3, 7, 9, 10, and 12) and 29 fiber QTLs associated with the D-subgenome (chromosome 14Lo, 20, and the long arm of chromosome 26) were reported in 2005 [12]. RFLP's were extensively used in identifying a wide-range of QTLs linked to fiber quality, length, strength, uniformity, wall thickness, micronaire, fineness, and maturity [13, 14]).

A second group of molecular markers, AFLPs, are powerful and efficient. They are being continuously used in cotton genomic investigations [15]. In the recent past, AFLP markers have been used in monitoring the differential expression of cotton fiber transcripts during elongation and secondary cell wall thickening in interspecific (*G. hirsutum* × *G. barbadense*) RI lines [16].

SSRs are the most informative, versatile and readily detectable DNA-based markers [17]. They have been used to determine agronomically and economically important genes, genetic linkage mapping, and linkage disequilibrium-based association studies in cotton [18, 19]. Although, traditional methods of microsatellite marker development are costly and time-consuming [20], large numbers of SSRs have been used to explore the molecular diversity, population structure and elite alleles of several Upland cotton cultivars [21–24]. As a result, many fiber quality traits associated marker loci and fiber development have been identified [21–23, 25].

3. Mapping for fiber-related traits in cotton

The genetic improvement in cotton with full utilization of conventional breeding has provided significant progress, but the complexity of the genome and limited understanding of economically important traits has deterred efficient breeding. Cotton molecular breeding has been a reliable tool for the characterization and mobilization of complex QTLs of interest. The advent of genomics-assisted breeding has become an effective method for selecting parents for agronomic, stress-responsive and fiber-related traits. In the past decade, focus of cotton genomic research has shifted from a few marker genotyping based QTL-mapping efforts to large-scale marker-based genome-wide association studies (GWAS), using high-throughput next-generation sequencing (NGS)-based genotyping methods.

An inter-specific high-density linkage map of allotetraploid cotton has been constructed using the F_2 population of *G. hirsutum* and *G. darwinii* with 2763 markers associated with 26 linkage groups covering 4176.7 cM genome and displaying a few differences between A_t and D_t subgenomes [26]. Among 601 distorted SSR loci reported [27], a lower number of segregation distortion loci were located in the A_t-subgenome than the D_t-subgenome. Recently, 185 cotton genotypes were evaluated for mapping of major fiber traits using 95 polymorphic SSRs [27]. This map also covered some other marker-trait associations, such as average boll weight, and gin turn out percentage together with fiber traits such as micronaire value, staple length, fiber bundle strength, and uniformity index. The results showed a clear association of MGHES-51,

MGHES-31 and MGHES-55 with all these traits, which is useful in future marker-assisted breeding and gene cloning studies in cotton [25, 27]. Similarly, MGHES-31 and MGHES-55 EST SSR markers were associated with lint percentage in a unique RIL populations derived from linted and lintless genotype crosses [21].

The SSRs have been used to examine molecular diversity, population structure, and to scan for polymorphisms. Genome-wide mapping in over 500 inbred cotton cultivars collected from China, United States, and the Soviet Union helped in identification of 494 fiber-quality-associated SSRs. Of the 216 markers related to fiber quality identified in this study [23], 13 were reported in other studies as fiber-trait-associated markers.

Gene-based markers were developed based on candidate genes and EST sequences to detect polymorphism in *Gossypium* species and for genetic and physical mapping. EST-derived microsatellites have been used to increase the number of microsatellites available for genetic map construction, and facilitated the use of functional genomics to elucidate fiber development process. EST-derived SSR-based high-density genetic maps for cotton fiber genes were reported in a number of studies [28–32]. The early EST-SSR studies in cotton [28, 29] focused on mapping loci in an interspecific (*G. hirsutum* × *G. barbadense*) RIL population while recent efforts aimed at mapping colored fiber loci (Lc 1 and Lc 2) [32].

With the advance on molecular marker development in cotton, three new marker types, indel (insertion-deletion), SNP, and retrotransposon-microsatellite amplified polymorphism (REMAP) were used to increase map density in allotetraploid cotton cultivars [33]. SNP markers in cotton can be used to associate genes with the respective fiber traits. Over one hundred genomic regions have been identified by tagging >2500 SSR and SNP markers using interspecific recombinant inbred lines (RILs) [34]. A large set of gene-associated SNPs were identified by comparative transcriptome profiling of four wild (*G. tomentosum*, *G. mustelinum*, *G. armourianum*, and *G. longicalyx*) and two cultivated cotton species (*G. barbadense* and *G. hirsutum*) [35]. By combining RNA-Seq and super bulked segregant analysis sequencing (sBSAseq) approaches, Li_2 mutant and its wild-type near-isogenic line (NIL) *G. hirsutum* cv. DP5690 were screened to identify the *Ligon-lintless 2* (Li_2) gene sequence, and subgenome-specific SNPs were identified in the tetraploid cotton [36].

Expansin protein plays an important role in fiber length and quality. Using NGS approaches, SNPs linked to six expansin genes were identified [37]. An α-type cyclin-dependent kinase (GhCDKA) protein has conserved cyclin-binding, ATP-binding, and catalytic domains. It plays a key role in fiber development. The *CDKA* gene expression was validated by northern blot and qRT-PCR analyses. Further, SNPs linked to CDKA gene locus was assigned to the chromosome 16 [38]. Using comparative and resequencing analyses, 24 million SNPs were identified between the A- and D-genomes in cotton. This analysis revealed that A-genome is relatively more variable (duplications and deletions) than the D-genome. In *G. hirsutum*, 1472 conversion events including 113 overlapping genetic events were identified between homologous chromosomes [39].

Two hundred twenty and 115 BAC contigs for two homoeologous chromosomes 12 and 26 of Upland cotton have been identified within 73.49 Mb and 34.23 Mb in physical length. Numerous fiber unigenes and non-unigene locations were found in both chromosomes [40]. New marker loci and linkage groups were applied in different cotton species using informative sequence-based markers and DNA sequence information. Some of the ESTs and BACs

were physically anchored and clustered into the high-density genetic map, and functionally annotated and classified into different *Gossypium* species [33].

A single dominant *Ligon lintless-2* (Li_2) gene results in significantly shorter fibers than a wild type in cotton (*G. hirsutum*). Two near-isogenic lines of Li_2 cotton (one mutant and one wild type) lines were painstakingly created by five backcrosses (BC_5) generations. An additional cross was used to develop a linkage map of the Li_2 locus located on chromosome 18. The SSR marker NAU3991 was successfully mapped and showed complete linkage with the Li_2 locus [41]. Marker-assisted breeding and *in vitro* mutagenesis of cotton ovules can provide an additional insight into the regulatory aspects of the li_2 mutation in cotton [42]. Similarly, using linkage mapping and analysis in *G. raimondii*, the Li_1 gene on chromosome 22 was identified. Many genes were recognized to be altered in the Li_1 mutant line for early termination of fiber elongation. Several additional studies have also identified factors that affect *Li*-associated genes at the downstream position [43].

In addition, a comprehensive review of molecular markers, marker-assisted selection (MAS) and marker-assisted backcross (MAB) breeding have been presented recently [44]. Cleaved amplified polymorphic sequences (CAPS) and derived-CAPS (dCAPS) markers obtained from the genes of interest are becoming increasingly valuable markers in molecular breeding of crops along with SNP markers. Phytochrome gene-based CAPS, transcription factor (*HY5*), and specific dCAPS in cotton were developed using comparative sequence analysis of the *PHYA1*, *PHYB*, and *HY5* genes that showed close association of these genes with fiber quality and early flowering traits in cotton [45, 46].

One significant QTL can control multiple fiber quality traits, such as fiber length, micronaire, strength and uniformity, and helps to elucidate the molecular basis of fiber quality. One such fiber QTL was found and mapped between HAU2119 and SWU2302 markers in a *G. hirsutum* RIL population. Three candidate genes have been identified within this QTL by RNA-Seq and RT-PCR analysis [47].

Recently, 352 wild and domesticated cotton accessions were screened, and 93 domestication sweeps have been assigned to 74 Mb and 104 Mb of the A- and D-subgenomes in allotetraploid cotton, respectively. Further, genome-wide association study (GWAS) has identified 19 candidate loci for fiber-quality-related traits. This study suggested possible asymmetric subgenome domestication for directional selection of long fibers. The effects of domestication on cis-regulatory divergence were shown by genome-wide screening for DNase I-hypersensitive sites and linking the variation to gene function [48].

Numerous additional genetic and genomic resources in cotton are available for the cotton research community through specific databases. In this context, CottonDB, established in 1995, is one of the earliest plant genome databases developed [49]. The International Cotton Genome Initiative (ICGI) was formed to coordinate the development of cotton genomics science including the creation of an integrated and saturated genetic and physical map in cotton [50]. The Cotton Microsatellite Database (CMD), an invaluable resource for cotton microsatellites, was developed to meet the goals of ICGI with the support received from Cotton Incorporated [51]. Later, the more comprehensive and robust database covering the genomic, genetic and breeding information collected from cotton was formed as CottonGen [52] database,

#	Author names	Publication year	Mapping population	Trait types mapped	Published journal	Reference
1	Iqbal and Rahman	2017	Inbred cultivars and segregating biparental population	Lint	Frontiers in Plant Science	[24]
2	Adhikari et al.	2017	F2 and F2:3	Fiber quality	Euphytica	[114]
3	Wang et al.	2017	BC3F2, BC3F2:3 and BC3F2:4	Fiber length	Theoretical and Applied Genetics	[44]
4	Su et al.	2016	Segregating biparental population	Fiber quality	Scientific Reports	[115]
5	Wang et al.	2016	RILs	Yield and fiber	PLoS ONE	[116]
6	Nie et al.	2016	Inbred cultivars	Fiber quality	BMC Genomics	[21]
7	Shang et al.	2016	RILs and BC	Fiber quality	G3:Genes|Genomes|Genetics	[117]
8	Liu et al.	2016	F2 and RIL118	Fiber quality	BMC Genomics	[43]
9	Badigannavar and Myers	2015	F2	Fiber	Journal of Cotton Science	[118]
10	Wang et al.	2015	RILs	Yield and Fiber	PLoS ONE	[119]

Table 1. Recent studies in mapping of fiber-related traits in cotton.

which has enhanced features such as data visualization, mining, sharing and retrieval [34]. Currently, there are 103 maps available in CottonGen [52]. A few recent mapping studies for fiber-related traits in cotton are presented (**Table 1**).

4. Genome-sequencing efforts in cotton

The genome-sequencing endeavor in cotton has significantly advanced our existing genomic knowledge for the past decade. Available full sequence of cotton genomes should provide a better understanding of fiber and fiber-related traits. The initial efforts in cotton genome sequencing were started with the closest progenitor diploid species, *G. raimondii* (D5) and *G. arboreum* (A_2), and later extended to other tetraploid species, *G. hirsutum* $(AD)_1$ and *G. barbadense* $(AD)_2$. Currently, whole genome-sequencing efforts of the diploid progenitor species, *G. herbaceum* (A_1), are progressing under collaboration between Alabama A&M University and USDA ARS [53]. Genome-sequencing information from the diploid and tetraploid species may aid in developing genetically engineered cotton lines, with superior agronomic traits. Recent reports in whole genome analysis in cotton are summarized in a tabular form (**Table 2**).

Authors	Year of publication	Species	Sequencing platform	Assembler used	Assembled genome size	Predicted genes	Journal published	Reference
Sripathi et al.	Unpublished	*Gossypium herbaceum*	Roche 454 and Illumina Hiseq2000	ABySS, SSPACE and SEALER	~1.46 Gb	41,387	Unpublished	[55]
Li et al.	2015	*Gossypium hirsutum*	Illumina HiSeq 2000 platform	SOAPdenovo, SSPAC, and GapCloser	~2.17 Gb	76,943	*Nature Biotechnology*	[120]
Liu et al.	2015	*Gossypium barbadense*	Roche 454, Illumina Hiseq2000, PacBio SMRT	Newbler v2.3.	~2.20 Gb	77,526	Scientific Reports (Published online)	[57]
Li et al.	2014	*Gossypium arboreum*	Illumina HiSeq 2000 platform	SOAPdenovo	~1.94 Gb	41,330	*Nature Genetics*	[121]
Wang et al.	2012	*Gossypium raimondii*	Illumina HiSeq 2000 platform	SOAPdenovo and SSPACE	~0.58 Gb	40,976	*Nature Genetics*	[122]

Table 2. Genome-sequencing efforts in cotton.

Briefly, several key facts have been gleaned from the tetraploid cotton, *G. hirsutum* and *G. barbadense*, genomes-sequencing efforts. Using whole-genome shotgun reads, BAC-end sequences, and genotype-by-sequencing (GBS) genetic maps, the allotetraploid *G. hirsutum* TM-1 genome was sequenced [54]. It was subsequently assembled into ~2.56 Gb genome with 32,032 and 34,402 genes from A_t and D_t subgenomes, respectively. Comparative subgenome analysis revealed higher percentages of gene loss, disrupted genes, structural rearrangements, and sequence divergence in the A_t subgenome when compared to the D_t subgenome. This can be attributed to the evolutionary irregularities. However, no genome-wide expression dominances were found between the two subgenomes. It is important and should be noted that the A_t subgenome, with its positively selected genes (PSGs) for fiber improvement, and stress tolerance in the D_t subgenome, are tied to genomic signatures of selection and domestication [54]. Between the two allopolyploid cottons, *G. barbadense* is the preferred species for producing superior, extra-long fibers. The WGS analysis of *G. barbadense* suggested that the uneven genome-wide duplication was 20 million years ago (MYA) and pseudogenization 11–20 MYA might be a probable cause of genomic divergence. Further, based on sequenced genomic information, the role of a fiber-specific cell elongation regulator, *PRE1* (with A_t subgenome origin), conditioning extra-long fibers was revealed [55].

Since the completion of whole genome sequencing (WGS) of the nuclear genome of cotton, the focus has been shifted to chloroplast and mitochondrial genomes [56]. The complete cotton chloroplast genome is 160,301 bp in length with a total of 131 genes, of which 112 are unique genes and 19 are duplicated genes. The cotton chloroplast genome lacks *rpl22* and *infA*, and contains a number of dispersed direct and inverted repeats. The phylogenetic analysis of cotton

and 25 other completely sequenced angiosperm chloroplast genomes revealed strong relationships among *Malvales*, *Brassicales* and *Myrtales* within the rosids clades [57].

The complete mitochondrial (mt) DNA sequence of *G. raimondii* was assembled into a circular genome of (676,078 bp), and then compared and analyzed with other plants. The analysis showed 39 protein-coding, 6 rRNA, and 25 tRNA genes. Interestingly, almost all of the *G. raimondii* mitochondrial (mt) genome has been transferred to Chr1 within the nucleus. The phylogenetic analysis with the other related mt genomes of rosids showed that *G. raimondii* is closely related to *G. barbadense*. Similar to the plastid genome analysis, the phylogenetic analysis of mt genomes revealed *Brassicales* were closely related to *Malvales* in the rosids clades [1]. Sequencing and characterization of both nuclear and cytoplasmic genomes of the *Gossypium* species will enrich the knowledge used to identify fiber-related genes for the improvement of cotton fiber quality trait using modern genetic engineering tools [58, 59].

The whole genome sequences of *G. hirsutum* and *G. barbadense* have revolutionized molecular biology investigations in cotton. Knowledge gained from decoding the cotton genome has helped to improve our understanding of gene function to ultimately benefit growers with improved yield and fiber quality. It has provided an unprecedented opportunity to bridge the gap between functional and structural genomic research by using the reference sequences of the tetraploid cotton genome. Scientists are using new advanced technologies "to mine" for useful genes and understand the molecular processes of fiber development for cotton germplasm enhancement. For example, Paterson [60] reported that among 48 genes for which expression is upregulated in domesticated *G. hirsutum* fibers at 10 dpa, 20 genes show 10-fold enrichment relative to random genes. They are within QTL hotspot affecting length, uniformity, and short fiber content. Thirteen genes show 15-fold enrichment and they are in the homologous hotspot affecting fiber elongation and fineness. The reference tetraploid genome sequence revealed that non-random patterns of diverse data sets that are concentrated in the specific small regions of the At and Dt genome [60]. Having such enriched genomic data in hand, scientists are much closer to identifying the causal gene(s). For example, expression patterns of genes in *G. hirsutum* wild type and its near isogenic fuzzless/lintless mutant at the stage of fiber initiation were analyzed using the RNA-Seq data [61]. Recently, Chen et al. [62] reported that differential gene regulation causes the difference in the quality of fiber between *G. barbadense* and *G. hirsutum* based on integrated genome-wide expression profiling markers [62].

5. Fiber-related transcriptome and gene expression studies in cotton

The functions of cotton transcriptome were studied using multiple techniques such as comparative genomics, BLAST, Gene Ontology (GO) analysis, and pathway enrichment by Kyoto Encyclopedia of Genes and Genomes (KEGG). Some prominent fiber-related findings were found. In this section, we briefly summarize some of key findings on this regard.

To study key fiber development genes, fuzzless/lintless (*fl*) cotton mutants were considered to elucidate molecular mechanisms relevant to fiber length development [63]. Furthermore, different fiber developmental stages have been studied to understand comprehensive mechanisms of overall fiber development. For example, *G. hirsutum* wild type (WT) and its near

isogenic fuzzless/lintless (fl) mutant were used in comparative transcriptome analysis and microarray studies of developing ovules [64, 65]. In *fl* mutants, at the fiber initiation stage of fiber development, calcium and phytohormone mediated signal transduction pathways, biosynthesis of auxin and ethylene, and stress-responsive transcription factors (TFs) were downregulated, whereas researchers observed a strong downregulation of genes related to carbohydrate and lipid metabolisms, mitochondrial electron transport system (mETS) and cell wall loosening and elongation at the fiber elongation stage of development. A number of genes including *CELLULOSE SYNTHASES* and *SUCROSE SYNTHASE C* were down regulated in *fl* mutants at fiber initiation and secondary cell wall biosynthesis stages compared to the WT [64]. Interestingly, it was reported that some of genes related to phytohormone signaling and stress response, upregulated in the WT genotypes in the early period of fiber initiation, started their expression in the later period of 15 day of post anthesis (dpa) in *fl* mutants [64].

Comparative transcriptome analysis showed that only a few genes were differentially expressed in zero dpa ovules, and three dpa fibers. The importance of auxin signaling and sugar signaling pathways in modulation of different fiber developmental stages was studied using pathway studio analysis [65].

Another transcriptome analysis of *G. hirsutum* WT and its mutant *fl* ovules in fiber initiation and elongation stages has been implemented using high-throughput tag-sequencing (Tag-seq). Differentially expressed gene (DEG) analyses results revealed substantial changes in gene type and abundance between the wild type and mutant libraries. Most of the DEG in WT1/M1 and WT2/M2 libraries developed for the study of the fiber cell development included cellulose synthase, phosphatase, and dehydrogenase genes [66].

In order to identify important genes of Ligon Lintless-1 (*Li-1*) mutants during the secondary cell wall synthesis stage, high-throughput microarray technology and real-time PCR were employed. There were at least 2-fold differences in at least 100 expressed transcripts found during secondary wall biogenesis using transcriptome analysis. Expansin, sucrose synthase, and tubulin expression gene families were identified in *li-1* mutant. This signifies *Li-1* gene activities during later fiber developmental stages [67].

A comparative microarray analysis was used to study fiber elongation in two short fiber mutants, and their near isogenic WT to identify key genes or metabolic pathways. Energy production, increasing mitochondrial electron transport activity, and response-to-reactive oxygen-related genes showed higher expression in short fiber mutants than in wild type. At least 88 fiber elongation genes were identified that were not affected by growth condition [63].

Improving the defects in the fiber secondary cell wall (SCW) resulted in non-fluffy fibers, low dry weight, and fineness fiber in the *immature* fiber mutant (*im* mutant) of *G. hirsutum*. Lower cellulose content and thinner cell walls were found in *im* mutant than its near-isogenic WT line (NIL) TM-1 at the same fiber developmental stage. Sucrose content, an important carbon source for cellulose synthesis, was also significantly lower in *im* mutant than TM-1 during 25~35 DPA. Comparative analysis of fiber transcriptional profiling indicated that SCW biosynthesis was 3 days later in *im* mutant than TM-1. Cellulose synthesis, secondary cell wall biogenesis, cell wall thickening, and sucrose metabolism were associated with genes significantly upregulated in TM-1. Quantitative reverse transcription PCR (qRT-PCR) analysis

validated that carbohydrate metabolism had 12 related genes that were differentially expressed at the earlier transition. qRT-PCR also showed differences in the SCW biosynthesis stages of fiber development between TM-1 and *im* mutant, and the importance of the *im* regulatory gene functions in fiber SCW biosynthesis [68].

Human selection altered the phenotypic evolution of fiber development over long periods of selective breeding. This has been shown by comparative transcriptome profiling of developing fiber using RNA-Seq analysis. Over 5000 differentially expressed genes were found with a regulatory or participatory role in primary and secondary cell wall synthesis between wild and domesticated cottons, which arose from artificial selection [69]. Some 210,965 unigenes of more than 100 bp were obtained from 47.2 million paired end reads of the anthers of TM-1 through transcriptome sequencing. Many enriched genes were found in the processes of transcription, translation, and posttranslation as well as hormone signal transduction. In addition, the participation of transcription factor families and cell wall-related genes active during cell expansion and carbohydrate metabolism were analyzed [70]. To determine fiber elongation and cell wall differentiation, combined transcriptome and metabolome analyses were studied in *G. barbadense* and *G. hirsutum* cultivars. Results showed that 10–28 dpa *G. barbadense* fibers showed primary cell wall synthesis to support elongation, transitional cell wall remodeling, and secondary wall cellulose synthesis for continued fiber elongation. Deep sequencing of transcriptomes and nontargeted metabolomes of single-celled cotton fiber showed that the discrete developmental stage of transitional cell wall remodeling occurs before secondary wall cellulose synthesis begins in both genotypes. Among all 40,000 transcripts, expressed in the fiber and all of the cell wall-related transcripts, expression was similar between genotypes. However, cellulose synthase gene expression patterns were more complex than expected. Oxidative stress in fiber tissues was lower in *G. barbadense* when compared to *G. hirsutum*. Using deep-sequencing transcriptomes and nontargeted metabolomes, a transcriptional repression of lignification during cell wall synthesis was

#	Authors	Year of publication	Methods	Tissue used	Journal published	Reference
1	MacMillan et al.	2017	RNA-Seq	Fiber quality	BMC Genomics	[123]
2	Miao et al.	2017	small RNA-Seq	Fiber quality	PLoS ONE	[124]
3	Li et al.	2017	RNA-Seq	Fiber development	BMC Genomics	[125]
4	Hu et al.	2017	small RNA-Seq	Fiber yield	Plant Biotechnology Journal	[126]
5	Thyssen et al.	2017	RNA-Seq	Lint	Plant Journal	[127]
6	Naoumkina et al.	2017	RNA-Seq	Lint	Genomics	[128]
7	Ma et al.	2016	RNA-Seq	Fiber development	PLoS ONE	[129]
8	Naoumkina et al.	2016	RNA-Seq	Fiber development	BMC Genomics	[89]
9	Hinchliffe et al.	2016	RNA-Seq	Fiber color	Journal of Experimental Botany	[130]
10	Zou et al.	2016	RNA-Seq	Fiber development	Science, China	[131]

Table 3. Recent transcriptome analyses studies in cotton.

identified. The results implicated a positive contribution of the ascorbate-glutathione cycle in improving fiber length by the enhanced capacity of reactive oxygen species (ROS) [71].

Crossed and backcrossed inbred lines of *G. hirsutum* and *G. barbadense* have been developed to investigate fiber yield per acre. Using these experimental populations, a number of yield and yield component QTL co-localized differentially expressed genes (DEGs) and DEG-based SSCP-SNP markers have been identified [72]. Numerous (1486) DEGs were found from (BIL) population using a microarray-based comparative transcriptome analysis in 10 dpa fibers. In 24 yield QTL regions and 11 yield QTL hotspots, the 212 DEGs were mapped. Within the 7 lint-yield QTL, identified in the BIL population, additional 81 DEGs were mapped [72].

In another study, using the cotton EST sequences, available at NCBI, 29,547 and 19,578 unigenes were assigned to the D_t and A_t subgenomes of tetraploid cotton, respectively. Among these, the majority of the abundantly expressed genes played intricate roles in cotton fiber development [73]. Selected publication for recent fiber development-related investigations are summarized in **Table 3**.

6. Characterization of specific genes for cotton fiber

A cascade of genes is expressed during the stages of fiber development. Recently, several novel genes were identified. α-expansins are one such gene family, which are cell wall proteins that disrupt non-covalent bonds between wall components to facilitate cell wall extension. Six α-expansin mRNA encoding genes were isolated using a genomic library screen and PCR-based strategies. Four genes in the expansin gene family (*GhExp3-GhExp6*) are expressed in multiple tissues of cotton, and only two genes (*GhExp1* and *GhExp2*) showed expression in developing cotton fibers. *GhExp1* transcripts are highly expressed, while *GhExp2* transcripts were detected at low levels in the fiber. Of these two, *GhExp1* is relatively of greater importance to cell wall extension during fiber development [74].

Actin-depolymerizing factor (ADF) is also one of the important genes that modulates the polymeration and depolymeration of the actin filaments, and is also important for fiber development. *GhADF2*, *GhADF3*, *GhADF4*, and *GhADF5* genes, encoding ADF proteins, have been isolated from cotton (*G. hirsutum*) cDNAs. Bioinformatic analyses have shown the molecular evolutionary relationships of these genes, including their highly conserved status. Interestingly, *GhADF2* was predominantly expressed in the fibers and not in other tissues [75]. Downregulation of *GhADF1* in the transgenic cotton plants showed increased fiber length and strength as compared to the wild-type fiber. Transgenic fibers also contained more abundant F-actin filaments in the cortical region of the cells than control fibers. In transgenic fibers, the secondary cell wall appeared thicker and the cellulose content was higher than that of the control. This showed the critical role of *GhADF1* in the processes of elongation and secondary cell wall formation during fiber development [76].

Plants have a signaling system that mediates responses to environmental stimuli. Co-expression and preferential interaction between two calcineurin B-like (CBL) proteins and CBL-interacting protein kinase (*CIPK*) genes in the elongating fiber cells of *G. hirsutum* were determined [77]. Very specific characterization of a receptor-like kinase gene (*GhRKL1*), especially during secondary cell wall synthesis in the cotton fiber cells, has been studied. In cotton,

the location of *GhRLK1* products is considered to be in the plasma membrane. Additionally, the *GhRLK1* gene's function is thought to be in the signal transduction pathway, i.e., the induction and maintenance of active secondary cell wall formation during fiber development [78].

The *DET3* gene, which encodes subunit C of the vacuolar ATPase (V-ATPase), participates in Brassinosteroid-induced cell elongation. Seven candidates' expressed sequence tags (ESTs) were screened to analyze the function of *GhDET3* on the elongation of cotton fibers, and yielded detail data about this gene. Results showed that the amino acid sequence of *GhDET3* had high homology with *DET3* from *Arabidopsis*, rice and maize. Ubiquitous expression of this gene in all the tissues and organs has been detected by qRT-PCR analysis. The highest accumulation of *GhDET3* mRNA peaked during the fiber elongation stage (12 dpa), compared with the lowest level at the fiber initiation stage (0 dpa ovules) underscoring the vital role *GhDET3* plays in cotton fiber elongation [79].

One of the transcription factor genes in the *MADS*-box family has wide-ranging roles in many diverse aspects of plant development. The gene transcripts have been detected in developing cotton fiber cells and in other plant tissues. Alternative splicing results showed that transcripts may have altered cellular roles. This was due to encoded proteins with altered K-domains and/or C-terminal regions, and their subsequently variant proteins [80].

Reactive oxygen species (ROS) plays a prominent role in signal transduction and cellular homeostasis, as well as in plant cell development [81, 82]. However, growing and maturing cells encounter oxidative stress when resource imbalances occur [81]. In order to study oxidative stress-related genes and their expression levels in a single plant cell, microarray analyses have been conducted [81, 82]. Antioxidant genes were substantially upregulated in domesticated diploid and polyploid cotton (*Gossypium*) in contrast to WT. In contrast, no significant influence was shown on regulation of ROS-related genes with genomic merger and ancient allopolyploid formation in three wild allopolyploid species. The ROS-related processes were regulated by different sets of antioxidant genes. [81]. Reduced expression of ROS gene has also been observed in *im* mature fiber mutants [83].

Ten cotton class III peroxidase coding (*GhPOX*) genes were isolated from *G. hirsutum*. Class III peroxidase, an heme-containing enzyme, is encoded by a large, multigene family. These genes participate in the release or utilization of ROS. Among them, *GhPOX1* showed the most predominant expression in fast-elongating cotton fiber cells, and transcription level of *GhPOX1* was over 400-fold higher in growing fiber cells than in ovules, flowers, roots, stems and leaves. Results suggested that *GhPOX1* plays an important role during fiber cell elongation, possibly by mediating production of ROS [82].

GhPFN1 is a gene isoform that was found to be preferentially expressed in cotton fibers. *GhPFN1* was also found to be tightly associated with the fast elongation of cotton fibers. Overexpression and quantitative analyses also confirmed that *GhPFN1* may play a critical role in the rapid elongation of cotton fibers by promoting actin polymerization [84]. Brassinosteroids (BRs) promote fiber elongation. The *BIN2* gene is a member of the shaggy-like protein kinase family, and functions as a negative regulator of BR signaling in *Arabidopsis*. Cotton *BIN2* genes have been characterized for investigation of BR-mediated responses in the development of cotton fibers. To further elucidate their role, cotton *BIN2* gene was

transformed into the *Arabidopsis* genome. Resulting transgenic *BIN2*-mutant *Arabidopsis* plants exhibited reduced growth, confirming the role of *BIN2* genes. The *BIN2* gene thus encodes functional bin2 isoforms that inhibit growth by negative regulation of BR signaling [85].

One approach used to detect novel genes during fiber development is by identification of fiber-associated gene-rich islands on cotton chromosomes. For this purpose, 10 gene-rich islands have been found in different stages of *G. hirsutum* fiber development on chromosomes 5, 10, 14, and 15 [86]. Distributions of a large number of fiber genes across the A_t and D_t subgenomes of AD tetraploid cotton have been studied extensively. In an attempt to develop an integrated genetic and physical map of fiber development, 103 fiber transcription factors, 259 fiber development genes, and 173 expressed sequence tag-short sequence repeats (EST-SSRs) have been mapped [87]. According to this study, major transcription factor genes and more fiber QTLs were mapped to the D_t subgenome than the A_t subgenome, whereas more fiber development genes were mapped to the A_t subgenome than the D_t subgenome. The D_t subgenome may provide the transcription factor genes that potentially regulate the expression of the fiber genes in the A_t subgenome [87].

Differential gene expression of candidate genes between wild type and mutant during fiber elongation stage has been shown by RNA-sequencing and qPCR analysis [88]. Twelve candidate genes of the Ligon lintless-1 mutant (*li-1*) were found in F_2 mapping populations derived from the cross of Li 1 and H7124 genotypes. In *li-1* mutant genotype genes encoding ribosomal proteins, actin protein, ATP synthase, and beta-tubulin 5 were found as a putative candidates impacting fiber development process [88].

Heat shock transcription factors (Hsfs) have an important role in both plant stress and development. Due to global warming, cotton is also experiencing increased exposure to elevated temperature. Consequently, the development of the yield and the quality of lint are affected. Forty *GhHsf* genes were selected by Wang et al. [84] that were characterized into three groups: A, B, and C. In cotton, these *GhHsfs* were observed in the majority of the plant tissues, particularly around the developing ovules. Exposure to high temperature in cotton plants showed that *GhHsfs39* demonstrated the most rapid response to the heat shock. It has been suggested that a differential expression of *Hsfs* may thus play a role in the fiber development that requires further study [89].

UDP-glucuronosyltransferase gene is a cytosolic glycosyltransferase that catalyzes the transfer of the glucuronic acid component of UDP-glucuronic acid to a small hydrophobic molecule. UGT makes up one of the largest and most important multigene family in plants. The cotton UGT, *GhGlcAT1*, gene promoter contains specific transcription regulatory elements, and provides clues about the roles of *GhGlcAT1* in cotton fiber development, especially during fiber elongation [90]. A phylogenetic study of the UGT proteins of cotton was studied in selected cultivars and wild *Gossypium* species. The study identified, analyzed, and compared 142 UGTs in *G. raimondii*, 146 in *G. arboreum*, and 196 in *G. hirsutum*. The conserved consensus sequence had 44 amino acids. It additionally showed a possibility of regrouping the *GrUGTs* and *GaUGTs* into 16 phylogenetic groups (A-P) and *GhUGTs* into 15 groups. Additionally, RNA-Seq data was used to study the expression patterns of the UGT genes in *G. hirsutum* wild type and its isogenic fuzzless/lintless mutant during fiber initiation [61].

WRKY gene products aid in managing stress responses in multiple plant species but they have not been extensively studied at various stages of fiber development. Ding et al. [87]

identified the relation between *WRKY* transcriptome factors and fiber development of *G. raimondii* and *G. arboreum* by studying their genome and transcriptome of 112 *G. raimondii* and 109 *G. arboreum WRKY* genes. The transcriptome analysis identified several *WRKY* genes active during fiber initiation, elongation, and maturation with different expression patterns between species. The association of *WRKY* allelic gene expression (D_t and A_t) in *G. hirsutum* and alternative splicing events were likewise seen in both diploid and tetraploid cotton during the developmental stage of the fiber. In summary, this study provided new results for the evolution and role of *WRKY* gene family in cotton species [91].

The role of the *MYB* family transcription factors was evaluated during the developmental stage of cotton fiber. Within 1986 *MYB* and MYB-related putative proteins, 524 non-redundant cotton *MYB* genes were identified and regrouped into four subgroups (*1R-MYB*, *2R-MYB*, *3R-MYB*, and *4R-MYB*). In addition, *MYB* transcription factors were classified into 16 subgroups according to the phylogenetic tree analysis. After analysis, 69.1% of all *GhMYBs* genes were identified as *2R-MYB* subfamily. Conclusively, this study highlights important aspects regarding the functions of *MYB* transcriptome factors in cotton fiber development. Furthermore, it contributes to the understanding of the regulatory network of *MYB* in affecting other functions of cotton fiber development [92].

CrRLK1L, one of the receptor-like kinase (RLK) gene family subgroups, has previously been demonstrated to be important in the development pattern and spatial regulation in cotton. *CrRLK1L* family is believed to act as sensors for the integrity of the cell wall and regulators of polar elongation. This study focuses on *CrRLK1L* in cotton fiber development. A total of 44 *CrRLK1L* genes were isolated from *G. raimondii*, 40 from *G. arboreum*, and 79 from *G. hirsutum*. Among these, six genes played an important role in fiber development [93].

To visualize PME expression levels, 80 PME genes (*GaPME01-GaPME80*) were isolated from *G. arboreum*, 78 (*GrPME01-GrPME78*) from *G. raimondii*, and 135 (*GhPME001-GhPME135*) from *G. hirsutum*. The differences in the PMEs expression levels at the developmental stage of fiber was observed using qRT-PCR. Predominant expression in fiber was during the secondary cell wall thickening stage suggesting tissue-specific expression patterns in cotton fiber [94].

LPAAT is an enzyme from the Kennedy pathway in higher plants encoded by a multigene family. Recently, the role of modified-LPAAT gene (A_t-*Gh13LPAAT5*) in increasing the cottonseed oil content and fiber quality has been proposed by combining the genome-wide and transcriptome analyses [95].

7. Small RNA-mediated gene regulation studies in cotton

Small RNAs (including microRNAs, tasiRNAs, and piRNAs) are mainly 17–24 nucleotide long sequences that are scattered across the plant genomes and play an important role in regulating target gene expression via posttranscriptional and translational repression at different stages of plant development. The discovery of novel miRNA genes will help in understanding the key mechanisms associated with miRNA genesis and regulation of fiber development in cotton. Although the regulatory mechanisms of microRNAs and small non-coding RNAs were determined in overall plant growth and development [96], their specific role in the regulation

of fiber cell elongation and developmental processes were more widely elucidated primarily after 2008. The roles of small interfering and microRNAs in the development of the cotton ovule and fiber elongation were annotated by Abdurakhmonov and his colleagues [97], as a first attempt in fiber genomics. They identified three plant microRNAs (miR172, miR390, and ath-miR853-like) and demonstrated dpa-specific small RNA expression profiles during ovule development. These result suggested the complex dpa-specific small RNA regulation in ovule development covering 0–10 dpa fiber development stages [97].

Multiple approaches have since been developed to identify the role of small RNAs in fiber initiation and elongation. For example, a deep-sequencing approach was used to investigate global expression and complexity of small RNAs in wild type and fuzzless/lintless cotton ovules. Over 20 conserved candidate miRNA families, including their 111 members, were identified during fiber initiation and elongation. More than 100 unique target genes were predicted for most of the conserved miRNAs; two cell-type-specific novel miRNA candidates were also determined in cotton ovules [98]. More than 4 million small RNA sequences have been analyzed from fiber and non-fiber cotton tissues. Thirty one miRNA families, including 27 conserved and 4 novel miRNAs, have been identified from these tissues. In addition, 19 unique miRNA families were also identified representing 32 miRNA precursors. Seven families had been previously reported, and 25 new miRNA precursors have also been found.

The enrichment of siRNAs in ovules and fibers in small RNA metabolism and chromatin modification becomes active during fiber development [99]. A recent study identified 46 novel and 96 known miRNAs in elongating cotton fibers. They also found 64 differentially expressed miRNAs, and of those, 16 were predicted as novel miRNAs [100]. Several novel fiber miRNAs have been identified using high-throughput sequencing technologies during the secondary cell wall thickening stage in cotton [101]. Small RNA libraries were constructed from developing fiber cells of the short fiber mutants *Li-1*, *Li-2*, and their near-isogenic wild-type lines. Among 24 conserved and 147 novel identified families, four miRNAs revealed significant negative correlations with fiber lengths [96].

Earlier miRNA-specific DNA markers were developed and mapped in cotton to study the genetic variation of miRNAs and their putative target genes [98] A number of pre-miRNA and putative target gene primers have been examined and polymorphic loci were mapped on the total tetraploid cotton chromosomes. MiRNA-based sequence-related amplified polymorphism (SRAP) markers were used in order to map more miRNA loci. RT-PCR analysis revealed unique expression patterns across different fiber development stages between the parents in pre-miRNAs and putative target genes [102].

In *G. hirsutum*, ~300 miRNAs have been identified targeting over 3000 genes that possibly regulate stress responses, metabolism, hormone signal transduction and fiber development [103]. Among 79 and 46 miRNA families identified in *G. hirsutum* and *G. raimondii*, respectively, eight miRNAs were specifically related to fiber elongation and associated pathways such as calcium and auxin signal transduction, fatty acid metabolism and anthocyanin biosynthesis, and xylem tissue differentiation. In addition, one tasiRNA was identified and its target, *ARF4*, was experimentally validated *in vivo* [104].

Approximately, 10 million non-coding RNAs (ncRNAs) from fiber tissue of the allotetraploid cotton (*G. hirsutum*) were sequenced 7 days after flowering (DAF), to identify 24 nt ncRNA

as the dominant species, followed by 21 nt ncRNA, and 23 nt ncRNA. This study further screened ~560 miRNA gene loci and suggested the role of miRNAs in elongation and secondary cell wall synthesis stages of cotton fiber development [105].

8. Functional genomics and genome-editing technologies in regulation of fiber genes

Genome modification (GM) and genome-editing technologies (GETs) are invaluable in the discovery of genes of interest, and support functional genomics of many organisms, including cotton. Various GM and GETs have been developed to investigate regulation mechanisms of genes. One of the most commonly used GM approaches is RNA interference (*RNAi*) that drew its historic support from antisense technology in the discovery of gene structures and the functions of organisms. *RNAi* is a new emerging technique based on homology-dependent post transcriptional gene silencing, induced by double-stranded RNA (dsRNA). Associations of many important genes with fiber development were detected using *RNAi* [3]. Recently, considerable work has been done highlighting the suitability of this method in cotton improvement [106–108]. For example, modifying or regulating flowering time is arduous in a plant improvement program, but it is sometimes a critical step to produce novel varieties with high yield that are better adapted to a specific environment.

In a collaborative project, scientists from Uzbekistan and USA developed cotton plants with early flowering, higher yield, and improved fiber qualities when *RNAi* technology was applied to regulate phytochrome gene [108] (**Figure 1**). Considering the potential of this research, scientists have patented this technology. A number of novel RNAi cultivars were successfully fielded trialed in over 60,000 hectares in Uzbekistan [3]. This is the first time a major crop is developed through the new RNAi technology and has been planted in such a large area.

A majority of RNAi published studies have focused on the functional aspects of cotton fiber-related genes [3, 109]. Later, Wang et al. [110] characterized dihydroflavanol 4-reductase (*DFR*) enzyme that mediates the biosynthesis of two polyphenols (anthocyanins and proanthocyanidins (PAs)) in Upland cotton. In order to silence *GhDFR1* in cotton, *DFR* gene was cloned from developing fibers, and used for virus-induced gene silencing. The results show a significant decrease in accumulation of anthocyanins and PAs when *GhDFR1* is silenced. In addition, a high decrease of two PA monomers, (-)-epicatachin and (-)-epigallocatachin, occurred in *GhDFR1*-silenced plant fibers while two new monomers, (-)-catachin and (-)-gallocatachin, were present compared to control plants infected with the empty vector. *GhDFR1* contribution in the biosynthesis of anthocyanins and PAs in cotton has thus been confirmed [110].

Overexpression of an important gene in an alien/host genome can be useful to detect gene functions and structure. The cellular functions of the class I of TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factor *GhTCP14*, from Upland, cotton were characterized by Wang et al. [111]. According to their work, the main expression of *GhTCP14* gene was detected in fiber cells at the initiation and elongation stages of development, and its expression increased in response to exogenous auxin. Overexpression of *GhTCP14* in *Arabidopsis thaliana* enhanced initiation and elongation of trichomes and root hairs. Moreover, it affected root

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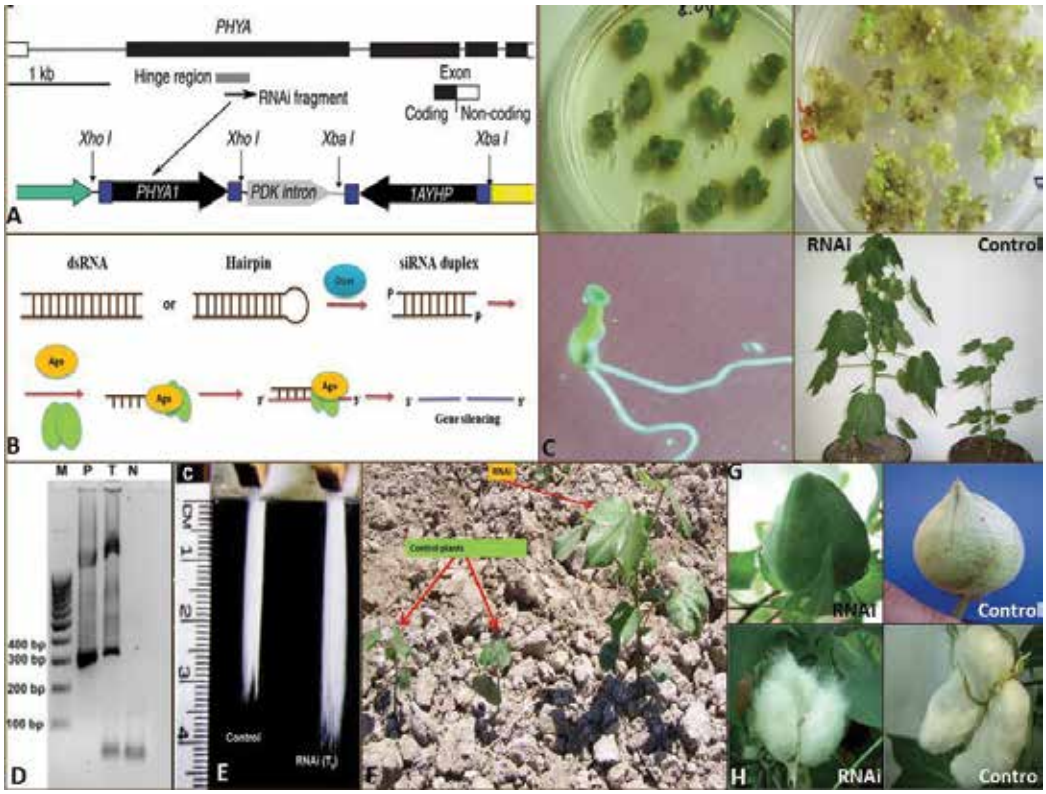

Figure 1. Different stages on the application of RNAi technology silencing *Phytochrome A* gene in *Gossypium hirsutum* grown at Uzbekistan (unpublished information collected from the reference 108). (A) Vector designed to introduce RNAi *PHYA1*; (B) RNAi mechanism in the cell; (C) *Agrobacterium tumefaciens* mediated cotton transformation with pHellsgate-8-*PHYA1* RNAi vector and plant regeneration using somatic embryogenesis through tissue culture system; (D) PCR-based marker confirmation of *PHYA1* RNAi regenerated cotton plant; (E) RNAiPhyA cotton line produces longer improved fiber compared to the control Cocker 312 plant; (F) RNAi plant showed rapid growth compared to the control at the young stage; (G) the cotton bolls showed different shape in control versus RNAiPhyA plant in the field; (H) matured bolls with fiber in RNAi Cocker 312 and control Cocker 312 cotton plants.

gravitropism like a mutant of the auxin efflux carrier PIN-FORMED2 (*PIN2*) gene. Expression of the auxin uptake carrier *AUXIN1* (*AUX1*) showed its upregulation, while *PIN2* was down-regulated in the *GhTCP14*-expressing plants. *GhTCP14* showed transcription activity by binding to the promoters of the *PIN2*, *IAA3*, and *AUX1* genes; these are auxin response genes that use electrophoretic mobility shift assays. All results demonstrated the potential regulation of *GhTCP14* gene in auxin-mediated differentiation and elongation of cotton fiber cells [111]. Overexpression of the actin-bundling protein *GhFIM2* was functionally characterized in cotton. The abundance of actin bundles is accompanied with accelerated fiber growth at the fast-elongating stage, and it increased when the *GhFIM2* gene was overexpressed. Secondary cell wall biogenesis also showed activation when the *GhFIM2* gene was overexpressed. These results indicated the importance of *GhFIM2* gene in the development of cotton fiber cells [112].

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (*Cas*) 9 protein system have emerged as a simple and efficient tool for genome editing in eukaryotic cells. Most of the commercially grown cotton is tetraploid, and it is much more

difficult to target both sets of homologous alleles. In an initial effort to standardize CRISPR/Cas9 in in the tetraploid cotton, a single copy gene, green fluorescent protein (GFP), has been utilized to determine the efficacy of the system in generating targeted mutations (indels) using three independent sgRNAs [113]. Literature analysis showed that application of novel generation GETs for cotton in general and fiber trait improvements in specific are in their very early stages and require more future attentions, coordinated efforts, and continuous investments.

9. Conclusion

For all crops—cotton in particular—where limited genetic diversity exists among the agriculturally elite types, genetic improvements will depend on innovative exploitation of genetic resources, and efficient strategies that effectively utilize both conventional and advanced molecular technologies. Comprehensive information is needed from independent and diverse research to understand molecular and genetic mechanisms associated with fiber development and additional agronomic traits. A cotton fiber provides a single cell crucible to understand the mechanisms of primary and secondary cell wall synthesis. It facilitates not only the study of cotton fibers, but also helps for further understanding of how all plant cell walls grow in relation to cell division and cell elongation. The elongating fibers also exemplify a scheme for production, and utilization of complex biochemical substances are first synthesized and then transported beyond the metabolic confines of cell membrane in the so-called "outer-space." A cotton fiber cell can be studied in detail relatively away from the noise of metabolic activity within the "inner-space" where thousands (and soon to be tens of thousands) of functionally characterized genes participate in a spatial and temporal interplay of plant growth. These genes first promote the regulated cell growth in organ-specific manner, and later march the specialized fiber cells toward senescence and apoptosis. To study the complex but very interesting process of fiber development, the cotton research community has extensively applied the genetic tools from mapping to genome modification technologies through characterization of key genes and development of molecular markers. This enabled researchers to tag complex fiber QTLs, clone and characterize genes and breed novel superior fiber cultivars using MAS and GE technologies. However, functional genomics of fiber is still behind other crops in the utilization of new generation native GETs due to complexity and multi-allelic nature of fiber-related genes, which require well-planned cooperative research activities and larger investments.

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Section 4

Transgenic Cotton and Biosafety Issues

Chapter 7

Transgenic Bt Cotton: Effects on Target and Non-Target Insect Diversity

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Additional information is available at the end of the chapter

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Abstract

Occurrence of diversity in ecosystem sustains particular characteristic of a biological community and also ensures stability of the community. Transgenic crops may affect insect biodiversity by unintended impacts on non-target arthropod population. For example, transgenic GM cotton specific to target lepidopterous pests can change the cotton pest spectrum and may induce the growth of new harmful pest species having no pest status. The change in species composition may influence IPM approach in cotton crop. The results of authors' research studies as well as global impact indicate that GM cotton is highly specific to target pests and has no unintended impact on non-target insect population. GM cotton provides significant season-long field control of target pests (*Helicoverpa armigera*, *Earias* spp. and *Pectinophora gossypiella*), with no significant control of *Spodoptera* species. The decreased insecticide use in GM cotton has a positive impact on beneficial insect populations and can increase the stability of rare species. Bt cotton has no resistance against non-target sucking insect pests. As GM cotton has no adverse effects on the non-target insect population and can reduce the use of broad-spectrum insecticides, it can become an important tool of IPM program in cotton agro-ecosystem of Pakistan.

Keywords: target insects, non-target insects, diversity, GM cotton, Pakistan

1. Introduction

1.1. Transgenic Bt cotton

Cotton plant has been genetically modified to incorporate gene conferring insecticidal protein (Cry1Ac) derived from the naturally occurring soil bacterium *Bacillus thuringiensis* (Bt) var.

Kurstaki. Genes that express the delta-endotoxins are called "cry genes [1]. In lepidopterans, the chewing mouthparts promote the ingestion of Bt toxins and the crystals are solubilized in the midgut having alkaline environment (pH 9 to 12). The crucial step in the activation of crystal proteins is the cleavage of toxins which may vary in different insect species [2]. Larvae stop feeding after Bt toxin ingestion due to the onset of paralysis in midgut, altered permeability and disintegration of the epithelium that leads starvation to death of the insect within 2–3 days after exposure. The larval death may vary depending on insect species, larval age and the amount of toxin ingested [3].

Monsanto developed and commercialized the first insect-resistant transgenic GM cotton expressing Cry1Ac gene (Bollgard® I) in 1996 [4]. GM cotton, the first transgenic non-food crop, has provided a specific, safe and effective tool for the control of lepidopterous pests [5–8] as compared to insecticides (pyrethroids and carbamates) that adversely affect non-target arthropods and other invertebrates [9].

Transgenic Bt cotton has provided an important tool for developing an integrated pest management (IPM) strategy [10, 11], especially for lepidopterous larvae in cotton [12–15]. GM cotton expressing Cry genes is cultivated on 33.1 million ha in different cotton growing countries including United States [16, 17], China [18–20], India [21–26], South Africa [27–29], Mexico [30], Argentina [31, 32] and Pakistan [33–43] and experienced many benefits like reduced use of broad-spectrum insecticides, improved control of target pests, reduced production cost, increased yield and better opportunity for biological control.

The targeted pests have developed the resistance against Bollgard I in most of the countries. To overcome this issue, Monsanto has released Bt cotton containing two Bt genes Cry1Ac and Cry2Ab (Bollgard II). However, there are some other alternative means to minimize the development of resistance in target pests including: a) planting of refuge crop that does not contain Bt based product for susceptible target insect pests, b) consistent and high level of expression of Bt proteins in all plant structures, c) monitoring for shift in baseline susceptibility of target pests to Bt based products, d) use of other IPM control strategies (sowing time, new chemistry insecticides etc.) [43].

1.2. Global status of GM cotton

It is estimated that there is a rapid adoption of GM crops globally (up to 30 countries), and almost 18 million farmers have been grown these crops on more than 2 billion ha. GM crops have reflected substantial economic, health, environmental and social benefits to farmers by increasing crop productivity and conserving biodiversity [44].

GM cotton is being planted in USA since 1996 and it is estimated that 93% of total cotton area (3.98 million ha) is under cultivation of Biotech cotton. Biotech cotton is the third most important GM crop in Brazil and estimated to occupy 1.01 million ha in 2016/17. In India, farmers increased the cotton productivity by planting GM cotton on 11.2 million ha representing 96% of cotton area. Paraguay approved GM Cotton in 2011 for commercial production, and keeping in view the benefit of this technology, about 12,000 ha was planted up to 2015–2016. In Pakistan GM cotton is being cultivated on 2.9 million ha (97%) of the total 3 million ha of cotton area [44].

1.3. Status of GM cotton in Pakistan

Adoption of Bt-cotton in Pakistan was not fast than that of the other major cotton growing countries. The cultivation of Bt-cotton in Pakistan started upon the release of Bt-cotton candidate lines (IR-NIBGE-2, IR-FH-901, IR-CIM-443 and IR-CIM-448, developed by NIBGE Faisalabad) in 2003–2004 for testing their performance in various localities of Pakistan. Later on these varieties started capturing area each year. In 2005–2006, area under these varieties was 0.20 million ha, of which 0.093 million ha was in the cotton belt of Punjab Province [45]. In 2009, Ministry of Food and Agriculture made a positive development for the introduction of Bt cotton varieties in the country to maximize cotton production and for this purpose a letter of intent was signed with Monsanto company, but process was delayed. During the meantime, these cotton varieties including IR-NIBGE-2 (approved as IR-NIBGE-1524), IR-FH-901 (approved as IR-NIBGE-901), IR-CIM-448 (approved as IR-NIBGE-3701) and Bt-121 acquired >40% of the total cultivated area of cotton in both the province (Sindh and Punjab). Later on these cotton varieties along with some new varieties were approved by the Punjab Seed Council (PSC) on March 31, 2010 to counteract the cultivation of adulterated and unapproved Bt cotton seed (**Table 1**).

Later on some more Bt cotton varieties were approved for commercialization but all these varieties contain a single Cry1Ac toxic gene. In 2014, Bt cotton was grown an area of 2.9 million ha indicating an adoption rate of 88% in the country. Of the approved 32 Bt cotton varieties, half were developed by private seed companies and half by public sector research institutes. It was estimated that about 700,000 resource poor and small farmers were benefited from Bt cotton cultivation. The economic benefits achieved from Bt cotton cultivation was US$1615 million for 2010–2013 [46]. However, the productivity of cotton in Pakistan is low (0.5 tons/ha) as compared to other Bt cotton growing countries. The agricultural productivity can be enhanced by increased adoption of Bt cotton, which would considerably

Sr. #	Variety/lines	Center of release	Year of cultivation and approval
1	IR-NIBGE-3701	NIBGE Faisalabad, Pakistan	Released for testing at farmer fields in 2003–2004 but approved in 2010 for Punjab, and in 2011 for Sindh
2	IR-NIBGE-901	NIBGE Faisalabad, Pakistan	Released for testing at farmer fields in 2003–2004 but approved in 2011 for Sindh
3	NS-121	Neelum Seed, Multan, Pakistan	Released in 2006, approved in 2010
4	MNH-886	Cotton Research Institute, Multan, Pakistan	Approved in 2012
5	FH-142	Cotton Research Institute, AARI Faisalabad, Pakistan	Approved in 2013
6	IUB-2013	Islamia University Bahawalpur, Pakistan	Approved in 2014

Table 1. The most popular Bt-cotton varieties (covered at least 10% area in any province) of Pakistan.

reduce insecticide applications, better quality of cotton, increased farm income, less exposure of insecticides to farmers and farm laborers and ultimate impact on food security efforts in the country.

1.4. GM cotton and insecticide use

Farmers rely heavily on the use of insecticides to control insect pests in cotton crop [47, 48]. This dependence on insecticides escalated the production cost. GM cotton containing Bt genes resulted in reduced application of pesticides for controlling the insect pests [8, 21, 49–51]. Insecticide application in Bt cotton has reduced up to 14 applications in China [52], 5-6 in Australia [53], 7 in South Africa [54] and 2.5 in India [55]. The introduction of Bt cotton in Southeast Asia significantly reduced the insecticide applications by 72%, increased yield of 11.4% and an estimated profit of US $126.02/ha [56]. The reduced insecticide use may increase the predator abundance and can affect the arthropod communities overall in Bt cotton field [57–61].

2. Diversity of insects on cotton crop

Cotton crop hosts a rich diversity of insect pests, predators and parasitoids. About 145 insect and mite pests have been reported in the cotton crop in Pakistan [97]. Cotton insect pests cause 35–40% yield loss [62]. The insect pest complex on cotton is divided into two groups: chewing insect pests and sucking insect pests. Among the chewing insect pests, cotton bollworm complex (*Helicoverpa armigera* (Hubner), *Pectinophora gossypiella* (Saunders) and *Earias Spp.*) are the most destructive ones in Pakistan and causes 30–40% yield reduction [63], because of damage to flowers, squares and bolls [64, 65]. Among sucking insect pests i.e., whitefly, jassid, thrips, aphid and cotton mealy bug are important [66, 67].

Farmers consider insecticides as a main sole to manage the insect pests in cotton crop. Most of the insecticides used, are broad-spectrum, which disturb the insect biodiversity, damage the beneficial insect fauna, hazardous to human health and environment, as well as leading to insect pests resurgence and outbreaks of secondary pests [68]. The insecticide application to cotton crop is the most intensive and the crop is to be considered as the largest insecticide consumer throughout the world [69]. It is estimated that in Pakistan, farmers spend US$300 million on pesticides annually, of which more than 80% is used on cotton, especially for bollworms.

2.1. Impact of GM cotton on target insect pests

Among the target insect pests of Bt cotton *Helicoverpa armigera* Hubner, *Earias* spp., *Spodoptera* spp. (Lepidoptera: Noctuidae), *Pectinophora gossypiella* Saunder (Lepidoptera: Gelechidae) and *Spodoptera* spp. are more serious pests of cotton in Pakistan. They damage the cotton plant by feeding on squares, flowers and bolls and in severe damage caused significant yield reduction [70].

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2.1.1. Cotton bollworm (Helicoverpa Armigera)

Commonly known as cotton bollworm (CBW) is one of the damaging pests of cotton and many other field crops worldwide [8, 71–73]. In India, this pest causes an estimate crop loss of US $350 million annually and farmers have to spray 15–20 times. Farmers in Pakistan also rely heavily on the use of chemical to control this pest and this indiscriminate use of insecticide particularly pyrethroids has developed resistance in this pest against insecticides [74, 75].

Our research studies have shown that transgenic Bt cotton offers great potential to significantly reduce the pesticide application for the control of major lepidopterous pest, *H. armigera* in Pakistan. The bollworm larval densities in Bt cotton remained below the threshold level; hence, no insecticide application is needed in Bt cotton. The results have shown no ovipositional differences between Bt and conventional cotton, as female moths cannot differentiate between Bt and non-Bt cotton for oviposition [76].

Transgenic Bt cotton varieties have lethal effect against *H. armigera* [77–81] and proved to be very effective in controlling this pest, causing 80–90% mortality in Australia [70], more than 90% in China [82] and 40–50% in India [83]. However, some studies have showed inadequate control of *H. armigera* with Bt cotton [84]. Some studies have showed no oviposition difference of *H. armigera* between transgenic Bt and non-Bt cotton [85, 86]. While, other reported greater number of eggs in Bt cotton than conventional cotton because of better leaf canopy due to lower damage [48]. It is also observed that there is a variation in Bt cotton resistance throughout the growing season and has shown the higher resistance to *H. armigera* at the last 10 days of May (94.5%) and July (83.3%), which decreased in August (22.7%) [84]. Similarly, some other field research studies conducted in Pakistan [87] and somewhere else [88–91] have showed significantly lower population of *H. armigera* in Bt cotton as compared to non-Bt cotton.

2.1.2. Pink bollworm (Pectinophora gossypiella)

It is the most important pest throughout the world, wherever the cotton is grown [92, 93] and almost difficult to control this pest because of its cryptic feeding habit. Bt cotton containing Cry1Ac can effectively control this pest [94–96]. Our research results indicated a lower density of rosette flowers and larvae in Bt cotton as compared to conventional cotton [97]. The study indicated that some larvae survived in Bt cotton, late in the season (end of September and October). It may be due to the decreased Bt toxin expression at lateral stage of plant [98]. However, it is admired that Bt cotton effectively suppressed the larval density in early season to an extent that pest could not cause an economic damage in the late season. Our results and those of other investigators support the efficacy of Bt cotton for pink bollworm control [99–101].

2.1.3. Spotted bollworm (Earias spp.)

It is an important pest of cotton in Indo-Pak subcontinent and cause damage to fruiting bodies and shedding of squares, flowers and bolls [102, 103]. Although, the primary target of transgenic Bt cotton is to control cotton bollworm, *H. armigera* but it also has a significant impact on other bollworm species, including *Earias insulana* & *E. vittella*. It occurs as an early

to mid-season pest in cotton and hence transgenic Bt cotton can effectively control this pest during early-mid phase of the crop, when toxin expression is high. Baseline susceptibility data has shown that Cry1Ac was highly toxic to spotted bollworm with LC_{50} ranged from 0.006 to 0.105 μg/ml of diet and 0.88 ng/cm^2 for leaf-dip bioassays [104].

Bt cotton containing Cry1Ac proved to be effective against this pest and significantly control the larval population [78, 105, 106]. Another research study conducted in Pakistan investigated the infestation trend of spotted bollworm in different plant parts of transgenic Bt and conventional cotton cultivars and reported minimum infestation of 3.36% in transgenic variety, "IR-FH-901" as compared to conventional variety, "FH-900" with 10.5% infestation [65].

2.1.4. Armyworm (Spodoptera spp.)

Commonly known as beet armyworm and fall armyworm is a multivoltine, polyphagous pest and can cause significant damage to cotton crop in case of severe infestation. Bt cotton with Cry1Ac proved not to be effective against armyworm, *Spodoptera* spp. [65, 105, 107–110]; hence, no significant differences in larval density between Bt and non-Bt conventional cotton [111, 112] and insecticide applications are needed to control this pest in Bt cotton. In Pakistan Bt cotton varieties proved to be less affective against armyworm and less mortality (13.3–53.3%) noted on different Bt cotton varieties containing CriAc. Some other field studies have shown that there were no significant differences in larval density among Bt and non-Bt cotton [112, 113]. As Bt cotton varieties expressing single toxin gene (Cry1Ac) have no resistance against armyworm, *Spodoptera* species, to overcome this problem a Bollgard® II cotton was developed that contain Cry1Ac and Cry2Ab, which provide the adequate control of armyworm and cotton bollworms [114–123].

2.2. Impact of GM cotton on non-target insect pests

The potential impact of GM crops on non- target organisms is a strategic concern among farmers, policy makers and scientist working on the development of GM crops as an ideal pest control tactic. Non-target organisms include all organisms except for the pest to be controlled. Examples of non-target organisms would be birds, reptiles, mammals, fish and other insects. A number of studies have shown that Bt toxin is highly selective and has no adverse effects on non-target insect fauna in cotton [124–127].

2.2.1. Impact of GM cotton on non-target major sucking insect pests

Among the non-target, sucking insect pests of GM cotton, whitefly, jassid, thrips, aphid and cotton mealy bug are the most important in Pakistan. These are very destructive pests during seedling and vegetative phase of cotton as they suck the sap of the plant, make it weak and in case of severe infestation wilting and shedding of leaves occur.

The field research study indicated that transgenic Bt cotton proved to be very effective against certain chewing lepidopterous pests and reduced the insecticide applications [37]. But at the same time, non-target sucking insect pests may become the significant insect pests, because the reduced use of insecticides in Bt cotton can increase the sucking insect

pest complex [90]. Most of the research studies have showed the higher population of sucking insect pests mainly; jassid, whitefly, aphid and thrips in transgenic Bt cotton [85]. Some other research studies conducted in Pakistan [63] and India [48] have found no significant differences in sucking insect pests; whitefly, jassid and thrips population among transgenic Bt and non-Bt cotton. As Bt cotton has no resistance against sucking insect pests and requires continuous use of pesticides and other control tactics for effective management of these insect pests [84, 105, 128].

Seed treatment provided the better protection against early-season sucking pests in transgenic cotton. As, there is no direct impact of Bt toxin on the non-target insect species but the ingestion of Bt toxin may prolong the development time during which herbivores are more exposed to parasitoids and predators [129]. It is suggested that Bt cotton along with pesticide applications could provide protection against target and non-target insect pests. But for the long term implementation of Bt cotton as a component of IPM, it is important that such varieties should be transformed with Bt genes that have also the resistance against non-target sucking pests to reduce the number of pesticide applications.

2.2.2. Impact of GM cotton on non-target natural enemies

Cotton crop hosts a rich diversity of insect predators and parasitoids, which have the significant role in regulating the pest population [130, 131]. Most of the field studies have shown no significant effects of Bt crops on natural enemies [40, 42, 60, 124, 129, 132–134]. Some reported the reduced activity of parasitoids in Bt cotton due to the absence of hosts or direct toxic effects of Bt toxin [86, 135, 136].

Bt cotton may act as a refuge for insect predators and spiders in large scale cotton production, where non-Bt cotton may be sprayed with insecticides [58]. Although Bt cotton is effective against target pests and have no direct influence on natural enemies [80] but there are the options that natural enemy population may be indirectly influenced by the behavioral change of non-target organisms or by the removal of their prey/hosts [124, 126, 137]. Some laboratory studies have reported indirect effects on natural enemies' population through unhealthy prey/hosts but at the same time population may be increased because of increased parasitism of unhealthy prey/host due to Bt toxin [124, 137–140].

Bt cotton can affect natural enemies in field by the removal of eggs, larvae and pupae of lepidopterous pests that serves as food sources [91]. Some studies showed the adverse effects of Bt toxin on the survival and development of some predators [109]. It may be due to the ingestion of Bt toxin during feeding on lepidopterous larvae or may be due to the consumption of intoxicated non-target prey that may pick up the Bt toxin [141]. While, most of the studies experienced no effect of Bt toxin on a main predator, *Chrysoperla carnea* [142] and reported no significant difference in abundance of insect predators between unsprayed Bt and non-Bt cotton fields [143]. The reduced insecticide use in Bt cotton can increase the predaceous arthropod population [144]. Some other field studies reported no significant difference of natural enemy populations between Bt and non-Bt cotton fields and where the differences were present, natural enemy populations were significantly higher in Bt than non-Bt cotton, mainly due to lower insecticide use in Bt cotton fields [145].

2.2.3. Impact of GM cotton on the overall abundance and insect diversity

Bt cotton can alters the insect diversity especially predators and parasitoids by reducing the abundance of *Helicoverpa* spp. and some other lepidopterous species [146–148]. A little numerical difference was found in the overall abundance and diversity of insect community in unsprayed Bt and conventional cotton fields [149], but another field study showed that species richness and total abundance reduced by 2.4–16.3 and 71.0–78.3%, respectively in Bt than non-Bt cotton fields [150]. Similarly, a three-year field studies have revealed no significant differences in species richness, evenness and diversity between unsprayed Bt and non Bt cotton, but plots receiving insecticides have slightly higher evenness.

The reduced insecticide use in Bt cotton may increase the minor insect pests' community, which are suppressed under intense insecticide applications [92]. The mirid bugs, which were minor insect pests in northern China, now have attained the status of main pests and population has increased 12-folds mainly due to the Bt cotton cultivation on large scale [151].

However, Shannon's index for total arthropod community and the neutral arthropod guild found significantly higher in Bt cotton fields than those in non-Bt cotton [152]. A comparison of Shannon-Weaver diversity indices in Bt and non-Bt cotton under sprayed and unsprayed conditions revealed that Bt cotton increased the diversity of arthropod communities and pest subcommunities; however, it decreased the diversity of natural enemy sub-communities [153]. A comparison of canopy and ground dwelling arthropod community revealed no significant difference in the abundance of total insect community between unsprayed Bt and non-Bt cotton [134]. In addition, the relative greater abundance of honey bees; *Apis mellifera*, *A. cerana*, *A. dorsata* and other pollinators in Bt than non-Bt cotton, indicate that Bt cotton may be a good source of nectar and pollen for insect pollinators [152]. Similarly, some other field studies have revealed that Bt cotton increased the stability of insect community, pest and natural enemy sub-communities and found no significant effects on the non-target insect diversity [154, 155].

3. Conclusions

A plenty of insects inhabit the cotton crop, including the target and non-target insects. Transgenic Bt cotton has resistance against major target insect pests; *H. armigera, Earias* spp. & *P. gossypiella* and significantly reduce the insecticide applications. This reduction in pesticide use has a positive impact on natural enemies and increased the stability of beneficial rare species. Bt cotton varieties with Cry1Ac toxin are ineffective against armyworm, *Spodoptera* spp. However, some inhibitory effects of Bt toxin on the growth of armyworm larvae are observed but there is a chance that this pest may become the major and alarming pest in Bt cotton field in Pakistan. Bt cotton has no resistance against sucking insect pests; jassid, whitefly, thrips, aphid & mealybug and insecticides are used to control these pests. To increase the stability of Bt based products as an important tool of IPM in cotton, it is crucial that such varieties should be transformed with *Bt* toxin genes, which also have other resistance traits against non-target insect pests to reduce the number of insecticide applications. There is also need to re-determine the economic threshold levels for sucking pests and bollworms in Bt cotton due

to increased beneficial abundance and the change of pest status. The biotechnological efforts, in developing the transgenic Bt cotton varieties, should also focus on the sustainable temporal and intra-plant expression of Cry1Ac toxin in all plant parts.

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Cotton, a source of natural fiber for textile industry, has a long breeding history aiming at increasing cotton fiber yield and its quality. Newly developed cotton varieties poorly respond in low-input environments. Secondly, the impact of changing climate may threaten the cotton production in the future. To address these challenges, efforts toward the development of resilient cotton varieties have been initiated using genetic and modern genomic approaches. In this book, research updates on cotton fiber types and properties, DNA markers for selecting desirable cotton plants, and cotton fiber genomics were compiled. Also, the modern breeding trends including development of transgenic cotton and the biosafety studies and possibilities of improving cotton genome using modern genome editing tools were also compressively discussed.

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