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Role of MicroRNAs and small RNAs in regulation of developmental processes and agronomic traits in *Gossypium* species

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ABSTRACT

Small RNAs (sRNAs) are short, non-coding, 17–24 nucleotides long RNA molecules that play vital roles in regulating gene expression in every known organism investigated to date including cotton (*Gossypium ssp.*). These tiny RNA molecules target diverse categories of genes from different bioliogical and metabolic processes and have been reported in the three domains of life. Small RNAs, including miRNAs, are involved in ovule and fiber development, biotic and abiotic stresses, fertility, and other biochemical processes in cotton species. Also, sRNAs are the critical components in RNA interference pathway. In this article, we have reviewed the research efforts related to the isolation and characterization of miRNAs using molecular and genomic approaches. The progress made in understanding the functional roles of miRNAs in regulation, alteration, and inactivation of fundamental plant processes and traits of importance in cotton are presented here.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding endogenous RNA molecules (sRNAs) that are 17–24 nucleotides (nt) in length. MiRNAs are evolutionarily ancient components and can serve as regulators of post-transcriptional gene expression in plants, animals, and some viruses [1, 2]. However, growing evidence indicates that the core components of the miRNA pathway are conserved between plants and animals [3]. In plants, miRNAs usually target mRNAs to induce gene repression through cleavage of the target transcripts. Whereas in animals, the miRNAs can recognize their target mRNAs with 6–8 nt at the 5′ end of the miRNA and block the translation process [4]. Essential roles of miRNAs in regulating gene expression in plants and animals have been elucidated by demonstrating their involvement in several biological processes such as stem cell differentiation, organ development, phase change, signaling, disease, and response to biotic and abiotic stresses [5]. Involvement of sRNAs and miRNAs in a variety of developmental processes such as cotton fiber and root development have also been studied [1, 6, 7].

MiRNAs can endogenously repress the gene expression either cleavage by perfect complementarity or translational inhibition by the partial complementarity of target mRNAs in plants and animals, respectively [8, 9]. First comprehensive screening of sRNAs in plants has been reported in *Arabidopsis thaliana* by utilizing a Sanger sequencing. Massively parallel signature sequencing (MPSS) has been used in the genome-wide discovery of sRNAs, and these are mostly encoded in the non-genomic locations of the genome [10]. A study of *Caenorhabditis elegans* with next-generation sequencing technology has identified 18 novel miRNAs and a novel class of 21 U-RNAs [11].

Research on the identification and characterization of sRNAs and miRNAs has been expanding due to the increased development and availability of multiple molecular and computational approaches [12]. The primary focus is on the role of these molecules in both growth and developmental processes as well as traits of agronomic importance. This

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review focuses on a broad spectrum of studies that have been conducted to date on the characterization of sRNAs and miRNAs in *Gossypium* species [12].

2. Approaches for the identification of microRNAs in cotton

Several molecular and computational methods have been developed and used to identify sRNAs and miRNAs in cotton species. Previous studies have demonstrated that the conservation of most miRNAs in diverse organisms; therefore, a strategy that leverages this conserved nature can be utilized for the identification of new orthologs by employing homology-based search methods. Using in silico approach, cotton ESTs and genomic sequences have resulted in the identification of twenty-two miRNAs belonging to 13 families, of which 7 miRNA families (miR160, 164, 827, 829, 836, 845, and 865) have been found for the first time in cotton that targeted transcription factors, cell division, or virus responsive genes [13].

In *G. hirsutum*, sRNAs of 18 to 26 nt length have been sequenced using high-throughput sequencing. Further analysis of 34 conserved miRNA families identified has demonstrated that only eight (miR156, miR157a, miR157b, miR162, miR164, miR393, miR399, miR827) of these possess coding loci that have been confirmed by screening cotton EST sequences [14]. Pang et al. [15] used miRNA-based AFLP marker analysis and compared the expression patterns of miRNA genes to evaluate the genetic variation among miRNA genes. Of the four miRNA genes studied (miR160a, miR171, 390a, and 396a), differential expression levels have been observed for three of them (miR171, 390a, and 396a) in early developing bolls of two cultivated cotton cultivars each belonging to *G. barbadense* and *G. hirsutum* [15]. Conservation of 33 miRNA families, with similar copy numbers and average evolutionary rates, have demonstrated in two closely related congeneric diploid species of cotton by the sequencing of sRNAs [16]. Besides, the functional divergence of conserved miRNA families has been demonstrated by the correlation between expression patterns of miRNA target genes and their controlling miRNAs [16].

A total of 446 miRNAs clustered into 224 miRNA families have been identified and characterized from the leaf tissues of Asiatic cotton, *G. arboreum* using transcriptome sequencing. Among these, 48 miRNA families have been conserved in other plant species, and 176 were novel. Of the novel miRNAs, gar-miR7504 showed the highest gene copy number followed by gar-miR166, gar-miR8771, gar-miR156, and gar-miR7484 [17].

3. The role of microRNAs and small RNAs in fiber development

Previous studies have demonstrated that the association of miRNAs in many aspects of cotton growth and development. Although the functions of miRNAs and sRNAs have been investigated in many *Gossypium* species, their involvement in the regulation of fiber cell elongation and developmental processes was not apparent initially. First attempts to reveal the participation of candidate small interfering RNAs/miRNAs in the development of cotton ovule has resulted in the identification of three plant miRNAs including miR172, miR390, and miR853 thus demonstrating the existence of sRNA expression profiles during ovule development that was specific to days post anthesis (DPA) [6].

A deep sequencing approach (DS) has been adopted, and various sRNA libraries have been constructed from wild-type and fuzz/lintless cotton ovules. To aid in investigating the global expression and complexity of the role of sRNAs during cotton fiber initiation and development, a total of 24 conserved and 147 novel miRNA families targeting fiber cells of the short fiber mutants, Li 1 and Li 2 and their near-isogenic wild-type lines using a degradome sequencing approach. Expression profiles of twenty miRNAs have also examined across a fiber development time in these plants and observed a significant negative correlation of 4 miRNAs and fiber length across 11 cotton lines [19]. The role of mutations in modulating the miRNA expression in fiber development has been proposed. The differentially expressed miRNAs identified in the Li 1 and Li 2 mutants will aid in better understanding of the regulatory mechanisms of cotton fiber development [19].

A total of 22 candidate miRNA families consisting of 111 members have been identified, of which, seven candidate miRNA families were abundantly expressed in the developing cotton ovule. Moreover, two cell-type-specific novel candidate miRNAs and 120 unique target genes for most of the conserved miRNAs have been identified in the cotton ovule. Results from this study have highlighted the importance of miRNAs in the regulation of fiber cell initiation and elongation, as previously reported [6, 18].

Another study identified 46 novel and 96 known miRNAs regulating cotton fiber elongation by utilizing sRNA DS. [20]. Further analysis of these novel and known miRNAs have demonstrated that 64 miRNAs (48 known and 16 novel) have differentially expressed during fiber elongation and development [20].

MYB transcription factors and phytohormone responsive factors are known to mediate the early stages of ovule and fiber development. However, these factors are targeted by miRNAs leading to the mRNA degradation or repression. To further gain insights into sRNAs, another study compared fiber and non-fiber tissues in cotton [21] and found 24 nt small interfering RNAs (siRNAs) as most abundantly enriched sRNA in ovules and fiber-bearing ovules when compared to leaves. In one of the cotton tissues examined, 31 miRNA families have been identified that included 27 conserved and four novel miRNAs. The abundance of miRNAs in ovule and fibers of cotton indicates the down-regulation of the putative targets through cleavage at the predicted sites and also suggest the role of RNA metabolism and chromatin modification in fiber development [21]. Such posttranscriptional changes result in the most elongated single cells in eukaryotes.

Another study that used quantitative real-time PCR (qRT-PCR) with eight cotton tissues from several developmental stages has demonstrated differential expression of miRNA classes and preferential expression of specific miRNAs in an organ-specific manner. Among all the miRNAs investigated, only miR-162 has been found to be highly expressed in immature fiber, 2 DPA ovules, and mixtures of 0 DPA stamen and carpel, which suggests its role in early stages of fiber development. Additionally, miR-396, previously implicated in the targeting of a fiber-related gene, callose synthase catalytic subunit has been expressed in all eight organs studied that included ovule and fiber suggesting its role in fiber development in addition to its function in other organs [22].

To investigate genetic variation in miRNAs and their target genes in cotton, Chen et al. [23] designed and surveyed specific primers based on pre-miRNAs and putative target genes as well as mapped nine pre-miRNA polymorphic loci and 156 target-gene specific polymorphic loci. Also, they created a network between miRNAs and their targets and provided comparative expression analyses of miRNAs and selected putative target genes between *G. hirsutum* and *G. barbadense* [23].

A study that combined high-throughput sequencing and computational analysis of sRNAs has confirmed the importance of sRNAs in the regulation of fiber elongation in *G. hirsutum* by confirming the expression of 79 known miRNA families in elongating fiber cells and predicting 257 novel miRNAs [24]. Targets of eight miRNAs were predicted using a *G. raimondii* genome, and their association with fiber elongation have been analyzed and experimentally validated, which corroborates the association of those miRNA targets with fiber elongation. Also, this study has identified one tasiRNA and its target, ARF4, which was experimentally validated in vivo using 5′-RLM-RACE [24].

The D genome sequence of *G. raimondii* and cotton EST sequences available from NCBI have been used as references for predicting miRNA precursors in a pool of sRNAs from two *G. hirsutum* libraries. They have been constructed to identify miRNAs involved in fiber initiation and seed development leading to the identification of 93 new miRNA precursors and 65 novel miRNAs [25]. Furthermore, this study obtained about seven hundred EST sequences which were targeted by candidate miRNAs [25].

MicroRNAs are involved in the regulation of fiber initiation and elongation, as well as in secondary wall thickening (SWT) and maturation. Liu et al. [7] have constructed seven RNA libraries from three fiber development stages (initiation, elongation, and SWT). Their study identified 47 conserved miRNA families and seven novel miRNAs. The expression of transcription factors, SBP and MYB, a leucine-rich receptor-like protein kinase, a pectate lyase, α-tubulin, a UDP-glucuronic acid decarboxylase and cytochrome C oxidase subunit 1, found to be suppressed by specific miRNAs during fiber development. Additionally, this study observed the biological activity of miRNA156/157 in ovule and fiber development. Further, it has demonstrated that suppression of miRNA156/157 could reduce the length of the mature cotton fiber [7].

Cotton is a model plant for cell fate determination, ploidy effects on gene expression and evolution of traits critically influenced the selection and domestication of crops. For example, MYB domain transcription factors are required for the initiation of leaf trichomes in Arabidopsis and lint in cotton. An investigation into the role of sRNAs has revealed that miRNAs contributed to the preferential degradation of homeologous mRNAs encoding MYB domain containing transcription factors [26]. To further expand our understanding of miRNAs, cotton is being used as a system for studying miRNA modification or degradation as truncation and tailing are critical for their functionality [27, 28].

From cotton seedlings and five developmental stages of cotton fibers, six sRNA libraries have been generated and sequenced. Their analysis has demonstrated that truncation or addition of only one or two nt on both 5′ and 3′ ends was the most observed modification in all six developmental stages and that truncation was more common than addition. Moreover, the structural analysis of 5′ and 3′ ends of miRNAs suggested that both ends were equally crucial for miRNA modification and that uridine was the preferred nt at the ends of both miRNAs and isomiRs. These results contribute to our evolving knowledge on the role of modifications of mature miRNAs and their regulatory function [28].

Transcriptome analysis of 10-DPA fiber in (PHYA1) RNAi plants was conducted by Miao et al. [29] to identify differentially expressed that have the direct impact on cotton fiber quality and were found in the RNAi line. The major classes of differentially expressed genes code for WRKY transcription factors, sucrose synthase, xyloglucan endotransglucosylase hydrolase, UDP-glucuronate: xylan alpha-glucuronosyltransferase as well as genes involved in lipid metabolism, and ABA/brassinosteroid signal transduction pathways. These results highlight the underlying molecular mechanisms of fiber improvement in the background of PHYA1 RNAi cotton line [29].

Although natural antisense transcripts (NATs) are commonly observed in eukaryotic genomes, only a small number of such NAT-generating genes are implicated in gene regulation in plants. The function of sRNAs derived from a NAT in fiber cell development was studied using a map-based cloning strategy in tetraploid cotton [30]. In which, the fuzz fiber development associated MYBMIXTA-like transcription factor 3 (MML3)/GhMYB25-like in chromosome A12, and GhMML3_A12 coding naked seed mutant gene (N1) was cloned to monitor the expression. The deficient expression of GhMML3_A12 in N1 has been associated with NAT production that was driven by its 3′ antisense promoter. They also observed that sRNA derived from the GhMML3_A12 locus could mediate GhMML3_A12 mRNA self-cleavage and lead to the production of naked seeds, followed by lint fiber inhibition in plants containing N1 [30].

4. MicroRNAs and small RNAs functioning in abiotic stresses

Many environmental factors influence the growth and development of cotton. For example, drought, salinity, heat, and chilling play a role in determining the fiber quality and yield in cotton. Genome-wide studies targeted to screen molecular mechanisms that regulate fiber and abiotic stress tolerant traits in cotton improved our existing knowledge of riboregulators in gene expression.

4.1. MicroRNAs associated with heat stress and drought

Rubisco activase regulates the mechanism of photosynthetic acclimation, and it has a suggested role in withstanding the heat stress in cotton [31]. Using sRNA and mRNA degradome sequencing, Wang et al. [32] studied low and high-temperature stress-responsive miRNAs and identified their corresponding targets. To investigate the effect of temperature stress, they exposed cotton seedlings with different temperatures (4 12, 25, 35, and 42 °C) and had identified a total of 319 known miRNAs and 800 novel miRNAs. Among which, 168 differentially expressed miRNAs between different temperature treatments and their targets have been identified. The majority of these gene targets were involved in biological processes such as response to hormone stimulus, oxidation-reduction reaction, photosynthesis, plant-pathogen interaction, plant-hormone signal transduction pathways based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [32].

To understand the role of miRNAs in male sterility two contrasting genotypes (HT-insensitive, 84,021 and HT-sensitive, H05) of cotton were exposed to high-temperature (HT) stress in cotton and identified 112 known miRNAs, 270 novel miRNAs, and 347 target genes from their anthers using sRNA and degradome sequencing [33]. Ding et al. (2017) found that miR156 has been suppressed in both HT-insensitive (84021) and HT-sensitive (H05) genotypes. Contrastingly, miR160 has been repressed in H05 and induced in 84,021 genotypes. Also, they identified that SPLs were induced in both 84,021 and H05 while ARF10 and ARF17 were induced in 84,021 but suppressed in H05 [33]. Further, they proposed that the miRNA-mediated auxin signal is necessary during cotton anther fertility when subjected to heat stress.

Long noncoding RNAs, (lncRNAs) are long transcribed RNA molecules (200 + nucleotides) that do not encode any protein. Lu et al. [34] identified 10,820 lncRNA from three different treatment conditions (control, drought stress and re-watering) in cotton using RNA-sequencing and bioinformatics. Of which, 9989 were lincRNAs, 153 have intronic lncRNAs, and 678 were anti-sense lncRNAs. Using functional analysis, they eliminated 6470 false positives (lncRNAs that encode proteins) and predicted 196 high confidence lncRNAs after screening for sRNA precursors. However, the lncRNAs were expressed at a low level when compared to other protein-coding genes. Their expression analysis suggested the role of lncRNAs under drought stress by regulating plant growth hormones [34].

4.2. MicroRNAs associated with salt stress

To understand the role of miRNAs in salinity, two cotton cultivars, a salt-tolerant, SN-011 and a salt-sensitive, LM-6 were subjected to salt stress [35]. Their differential expression analysis revealed four dramatically down-regulated miRNAs (miR156a/d/e, miR169, miR535a/b, and miR827b) while three miRNAs, (miR167a, miR397a/b, and miR399a) were up-regulated in the salt-tolerant cultivar, SN-011. Whereas only miR159 has been found to be down-regulated in the salt-sensitive cultivar, LM-6. Exploration of such differentially expressed miRNAs can contribute to our understanding of the role of transcriptome homeostasis in the adaptation to salt stress responses in cotton [35].

In a study that used comparative transcriptomics analysis to monitor gene and miRNA differential expression in leaves of salt tolerant and sensitive genotypes of cotton subjected to salt stress. A total of 108 conserved miRNAs have been found to be differentially expressed between salt-stressed and un-stressed conditions in the salt-tolerant genotype [36]. Majority of these differentially expressed and up-regulated genes encode for membrane receptors and transporters, biosynthetic and signal transduction pathways. Salt-stress response analysis of miRNAs and their target genes revealed that five of the nine target genes exhibited inverse correlations with their corresponding miRNAs. Thus, this study contributed to the deciphering regulatory pathways for the salt-responsive miRNAs [36].

A recent study reported miRNVL5 that targets GhCHR gene that plays a role in Na + accumulation in plants, seed germination, seedling growth, primary root growth, and biomass production of the cotton plant in response to salt stress by screening different developmental stages of cotton and transgenic Arabidopsis with GhCHR gene [37]. The expression of miRNVL5 repressed under different salt stress conditions (50 and 400 mM NaCl) with the concomitant higher expression of GhCHR in the cotton seedlings. This group identified an ortholog of GhCHR in Arabidopsis (At2g44380) and proposed a possible mode of action of miRNVL5 under salt stress using degradome analysis [37].

5. MicroRNAs small RNAs associated with biotic stresses

Understanding and elucidating the mechanisms of host-pathogen interactions are long-standing and trending goals of crop breeding and biotechnology. Among the many approaches to address these goals, identification and annotation of miRNAs are instrumental in understanding biotic stress induction and responses.

5.1. MicroRNAs and small RNAs associated with fungal diseases

Verticillium wilt (*Verticillium dahliae* Kleb), a soil-borne fungal pathogen causes yellowing, wilting, and eventually death in cotton. The early studies to observe the differential expression of miRNAs between verticillium tolerant (*G. barbadense* L., Hai-7124) and susceptible (*G. hirsutum* L., Yi-11) genotypes of cotton was reported by Yin and his group [38]. In which, they identified 215 miRNA families, of which over 65 miRNA families exhibited differential expression between verticillium tolerant and susceptible sRNA libraries. This study also found two trans-acting siRNAs and thousands of endogenous siRNA candidates in response to the Verticillium inoculation. Also, they made an exciting discovery that many siRNAs perfectly matched with the retrotransposons, suggesting the possible role of retrotransposons in the generation of endogenous plant siRNAs [38].

In another study aimed at the identification of miRNAs and their corresponding target genes involved in the regulation of Verticillium defense, identified 140 known and 58 novel miRNAs in cotton roots inoculated with *V. dahliae* [39]. Degradome sequencing and GO analysis indicated that both known and novel miRNAs had targeted many essential genes in root development and plant defense responses [39].

In a recent study conducted by another group aimed to understand the regulation of resistance-related genes identified 37 novel miRNAs by sequencing two sRNA cDNA libraries obtained from Verticillium-resistant Upland variety KV-1 after inoculating with this pathogen [40]. Using the criterion of no more than three sequence mismatches between the novel miRNAs and their potential target mRNAs. Among these, 24 novel miRNAs targeted 49 putative mRNAs/genes that are involved in plant-pathogen interactions, endocytosis, mitogen-activated protein kinase (MAPK) signaling pathway, and secondary metabolite biosynthesis. Some of the novel miRNAs and their corresponding target genes identified in this study has proposed role in promoting Verticillium wilt resistance [40].

A soil-borne fungus, *Fusarium oxysporum* f. sp. Vas infectum (FOV), another severe cotton pathogen that causes Fusarium wilt, resulting in significant yield losses. The fusarium pathogen is distributed worldwide and causes damage to several economically important crops. Although many studies to date have focused on the harmful effects of Fusarium and the physiological mechanism of wilting, very few reports are available.

To understand the molecular players underlying host defense responses, using a site-directed and adenylated linker-based sRNA cloning strategy followed by bioinformatics analysis, Shapulatov et al. [41] sRNAs from the cotton roots and identified miRNAs that were abundantly and expressed explicitly during FOV pathogenesis. Of 4116 candidate sRNA sequences identified from four complementary DNA (cDNA) libraries of non-infected and FOV (race 3)-infected roots of susceptible (B11970) and resistant (Mebane B-1) cotton genotypes (*G. hirsutum*), 4% of these matched with previously identified plant miRNAs. Further, a target analysis of the sRNAs identified proteins that were associated with fundamental biological processes and molecular functions pointed towards the demonstrated role of miRNA in host defense responses during FOV pathogenesis in cotton [41].

Zhu and his colleagues [42] investigated the conservation of regulatory relationship between miR482 and NBS-LRR genes in *G. hirsutum* and *G. raimondii*. The Upland cotton-specific miR482 efficiently targeted three of four NBS LRR genes of *G. raimondii* thus supporting the thought of cross-species gene regulation by miRNA, which has also been shown in tomato. It was proposed that the cleavage of NBS-LRR trigger the production of phased secondary sRNA in cotton. In *Verticillium dahliae* infected seedlings of the susceptible cultivar, Sicot71 (*G. hirsutum*) the repression of miR482 family of miRNAs (ghr-miR482b/miR482b.2, ghr-miR482c, and ghr-miR482d.2) resulted in corresponding NBS-LRR targets of in cotton, similarly as observed in tomato under fungal/bacterial infection, suggesting that the mode of miR482 regulation is conserved [42].

5.2. MicroRNAs and small RNAs associated with viral disease resistance

Using an in-silico approach, Shweta and Khan [9] made the first reported prediction of cotton miRNAs that have the potential to target cotton leaf curl Allahabad virus (CLCuAV) genes that facilitate viral replication and suppression of the host. They demonstrated that miR2950 of upland cotton could target all the viral genes, while miR408 targets were overlapping transcripts of AC1 and AC2 genes that code for replication associated protein (Rep) and transcriptional activator protein, respectively, in CLCuAV. Similarly, a set of miRNAs (miR394, miR395a, and miR395d) that had complementary sites on the transcripts of AC1 and AC4 genes have been identified.

Cotton leaf curl disease, caused by a begomovirus pathogen complex that include Cotton leaf curl Multan beta satellite (CLCuMuB) and Cotton leaf curl Multan virus (CLCuMuV) are the significant constraint to cotton production in South Asia. Viral sRNA (VsiRNA) profiles from CLCuMV and CLCuMB in infected upland cotton (*G. hirsutum*) plants have been obtained using DS. Computational analysis predicted hundreds of host transcripts that are potential targets of vsiRNAs, and some of these targets encode transcription factors that are associated with biotic and abiotic stresses. QRT-PCR analysis has implicated significant down-regulation of vsiRNA targeted gene in ClCuD-infected cotton. Using virus-induced gene silencing (VIGS) and 5′-rapid amplification of cDNA end (5′-RACE), the potential function of vsiRNA in CLCuD infection has been determined [43].

Viral diseases are a serious concern, and we have few control measures to reduce the devastating crop losses. A classic example is the cotton blue disease, which is prevalent in the southern part of South America. The etiological agent of the cotton leafroll dwarf virus (CLRDV) is accurately transmitted to host plants by the aphid vector, *Aphis gossypii*. The first report on the profile of sRNAs in a plant infected with a virus from the family Luteoviridae was reported by Silva et al. [44]. After the infection, Dicer-like (DCL) ribonucleases produce viral sRNAs (vsRNAs) from the viral genomes, which are then used as guide molecules for silencing its genome. The profiles and members of vsRNAs are poorly studied in the family Luteoviridae, a group of phloem-restricted viruses. Deep sequencing and analysis suggested that virus-derived double-stranded RNA (dsRNA) functions as one of the primary precursors of vsRNAs, and, based on the size classes profiled, it was apparent that most of the DCLs in cotton is responsible for silencing the virus.

Using DS, detailed characterization of sRNAs from the leaves of CLRDV-infected cotton plants indicated equivalent amounts of sense and antisense vsRNAs ranging from 21 to 24 nt long, with 22 nt class being the most abundant. This vsRNAs matched to viral genomes with higher frequencies of matches in the 3′ region. This study also showed general upregulation of cotton DCL transcripts during viral infection, except for DCL2, which has been found to be down-regulated [44].

Using DS of sRNAs in cotton leaves infected with Cotton leafroll dwarf virus (CLRDV), a Luteoviridae family; Romanel and his colleagues [45] were the first to explore global alterations of sRNAs in virus-infected cotton plants. Following the identification of 60 putative conserved cotton miRNAs, including 19 new miRNA families, their investigation has revealed that some of these miRNAs were mis-regulated during viral infection. Also, such alterations in the expression of miRNAs could be responsible for the development of disease symptoms during the infection. Further, they observed the quantitative and qualitative alteration of 24 nt long heterochromatin-associated siRNAs in the infected plant, leading to the reactivation of at least one cotton transposable element. The results from their study indicated that at least some of the virus-induced symptoms could be the result of deregulation of miRNA and epigenetic networks in the host plant [45], later supported by other studies.

5.3. MicroRNAs and small RNAs associated with insect resistance

Synthetic miRNA mimics are promising in controlling insect pests and prompted scientists to investigate them further. Zhang and his colleagues identified 127 conserved miRNAs in Beet armyworm (*Spodoptera exigua*), a significant pest of cotton worldwide. They used sRNA DS technology to find candidate miRNAs for controlling this pest [46]. The effects of 11 miRNAs from a pool of 127 miRNAs were tested on larval development of this insect. Overexpression of selected miRNAs including sex-miR-10-1a, sex-miR-4924, and sex-miR-9 via oral feeding has demonstrated the suppressed growth and mortality of *S. exigua*. Contrarily, the overexpression of sex-miR-4924 has resulted in a significant reduction in the expression level of chitinase 1, thereby causing abortive molting [46]. These results demonstrated the utility of miRNA in regulating insect development and thereby insect control.

5.4. MicroRNAs and small RNAs associated with nematode resistance

The root-knot nematode, *Meloidogyne incognita* is one of the most damaging plant-parasitic pests of many cash crops, including cotton (*G. hirsutum*). The whole genome sequence of *M. incognita* facilitated advancements in our understanding of the molecular interactions between this nematode and its plant hosts. As the central metabolic pathways and stress responses were found to be regulated by common miRNAs in *M. incognita* and many other organisms.

Using a bioinformatics tool mirDeepFinder, Zhang et al. [47] identified and analyzed miRNAs from a DS-generated sRNA database of *M. incognita*. They identified 254 conserved miRNAs from 161 miRNA families and 35 novel miRNAs belonging to 31 miRNA families. Their results also included 16 miRNAs that have been most abundant in their dataset and have also been found to be highly conserved across diverse taxa from helminths to vertebrates. Most importantly, a high level of conservation among nematode species including parasitic and infectious nematodes has been documented. Also, they identified several miRNA-regulated gene networks related to essential metabolism and stress responses. These results, along with those of similar studies, help elucidate the critical roles of miRNAs in unraveling the development and parasitic aspects of this economically important pest [47].

6. MicroRNAs and small RNAs in fertility, somatic embryogenesis and plant development

MicroRNAs and sRNAs not only regulate fiber development and stress tolerance but affect plant development and fertility as well.. SRNAs regulate almost all gene expression processes in cotton, including seed germination, flowering, fruiting, and maturity. A trait of utmost importance in utilizing heterosis in cotton is genetic male sterility (GMS). Despite many efforts, the underlying molecular mechanism of GMS is still unclear. Although several studies reported the role of miRNAs in flower and anther development had been unambiguously shown, their role in GMS has not yet been demonstrated.

Using DS to investigate expression and complexity of sRNAs during cotton anther development Wei et al. [48] observed distinct patterns of sRNA regulation in mutant and wildtype. A total of 16 conserved miRNA families have been identified, of which four miRNAs were highly abundant, while six conserved families were differentially expressed between GMS mutant and wild-type. Further efforts to identify different sets of miRNAs between GMS mutant and its wild-type improved our understanding of the regulatory mechanism for male sterility [48].

In a similar study, the involvement of miRNAs in regulating the male sterility has been investigated by sequencing sRNA libraries that were generated from a male sterile line Yu98-8A and the near-isogenic male fertile line, followed by bioinformatics and degradome analyses [49]. Their sequence analysis revealed 1588 and 1536 known miRNAs and 347 and 351 novel miRNAs, from male-sterile and male-fertile libraries, respectively. Among which, 49 conserved and 51 novel miRNAs have been found to be differentially expressed between MS and MF-lines. Further, their analysis revealed regulatory role of miR388 in during floral induction and development. Moreover, the qRT-PCR and plant phytohormone analyses have indicated that indole-3-acetic acid and gibberellic acid signaling transduction pathways play critical regulatory roles in male sterility [49].

Somatic embryogenesis (SE) is a significant phenomenon that determines cellular totipotency via dedifferentiation and re-differentiation resulting in the restructuring of embryogenic cells or somatic embryos, thus, regenerating into new plantlets. SE aids in crop improvement by increasing our understanding about the embryogenic competence, regenerability, and underlying regulatory mechanisms. Yang et al. [50] identified sRNAs and their targets during cotton SE, by using high-throughput sRNA, degradome sequencing and by comparing seedling hypocotyl and embryogenic callus of *G. hirsutum* YZ1. They identified 25 novel miRNAs and 36 known miRNA families differentially expressed between SE-induced and normal cells. Further, they opined the need for comprehensive efforts in profiling the miRNAs and their target genes to identify miRNA-target gene networks during cotton SE [50].

MicroRNAs and secondary small interfering RNAs (sec-siRNAs) are gaining importance as crucial regulators of post-transcriptional gene expression. Hu et al. [51] performed a bioinformatics analysis of sRNAs produced from leaves, flowers, and bolls of *G. arboreum*, to understand the biological significance of miRNAs and sec-siRNAs, to extend existing repertoire of sRNAs in diploid cotton species, and to further elucidate the role of sRNAs in tissue development in cotton and other species [51].

Studies on the functional analysis of miRNAs in plants demonstrated their relationship in the regulation of a wide range of developmental processes. Expression profiles of miRNAs have been investigated in four tissues of *G. raimondii*, a D genome model species of cotton to identify 16 miRNAs that play a vital role in plant growth and development [52]. Eight miRNAs (miR-159, miR-162, miR-164, miR-172, miR-390, miR-395, miR-397, and miR-398) showed tissue-specific expression and had been found abundantly in the flower buds, suggesting their role in the floral development. Contrarily, miR-164 showed tissue-independent expression pattern and had higher expression levels in flower buds and shoots, suggesting its functionality in both floral and shoot development processes. Whereas, miR-166 and miR-160 were highly expressed in all the four tissues under comparison, suggesting their regulatory roles in multiple developmental stages of the plant [52].

The fruit branch formation and flowering pattern are two critical characteristics that determine cotton yield and production. A nulliplex branch type is a rare phenotype resulted due to a mutation that can be genetically determined and is associated with a cluster branch trait in cotton. The pre-squaring stage is a crucial stage that controls the transition from vegetative to reproductive phase during the cotton growth and development. The differentially expressed genes (DEGs) and miRNAs have been identified between seedling, pre-squaring, and squaring stages in nulliplex and normal-branch types of cotton lines selected from *G. barbadense* and *G. hirsutum*. Their results suggested that DEGs were predominantly enriched in transcription factors that regulate branching and flowering in both *G. barbadense* and *G. hirsutum*. Thus, enumerating the cooperative regulation of fruit branch development and flowering induction and provided insights into the underlying molecular mechanisms [53].

7. Role of microRNAs and small RNAs in gene silencing processes

Involvement of miRNAs and sRNAs in the regulation of gene expression as well as their importance in gene silencing processes has been investigated extensively. Today, they are widely used as genetic tools for the targeted post-transcriptional regulation of the gene of interest. Short interfering RNA (siRNA) is a commonly used tool for studying post-transcriptional gene silencing (PTGS), for identifying the function of novel genes, and for validating drug targets in the pharmaceutical industry.

Studies that have used siRNAs for silencing expression of target genes are numerous. For example, in a study, Tang et al. [54] demonstrated that concentrations of chemical inducer dexamethasone in an optimal inducible system have shown varied gene expression among transgenic cell lines using green fluorescent protein gene (GFP) in cotton and other plants, by designing two siRNA constructs (siRNA-a and -b) against specific areas of the coding region in the same target gene. Gene inactivation mediated by siRNA can be highly specific to its targets, and therefore it is considered as a reliable molecular genetic tool for validating gene functions in different plant species [54].

Also, PTGS induced by siRNA is an efficient method for genetic and molecular analysis of specific developmental and physiological processes. Plus, different delivery options have been investigated for introducing siRNAs into cells. Nanosecond pulsed laser-induced stress wave (LISW) is a method that efficiently delivered siRNA for post-transcriptional gene silencing of a cotton gene [55]. Gene silencing induced by siRNA using LISW delivery method has been confirmed by northern blot, laser scanning microscopy, and siRNA analysis. LISW-mediated siRNA delivery is the most reliable and effective method for inducing PTGS in cultured cells [55].

Phytochrome A1 (PHYA1) RNAi lines of cotton, *G. hirsutum* L. cv. Coker 312 have been generated, by the silencing of PHYA1 gene using RNA interference [56]. Later, miRNA sequencing was conducted using a multiplex sequencing approach, and a total of 77 conserved miRNAs belonging to 61 families have been identified in a PHYA1 RNAi line and its parental Coker 312 genotype. Seven miRNAs (miR7503, miR7514, miR399c, miR399d, miR160, miR169b, and miR2950) were differentially expressed in PHYA1 RNAi cotton. Also, 35 novel miRNAs have been identified in fibers for the first time [57]. Involvement of the target genes for these differentially expressed miRNAs has been found in the metabolic and signaling pathways associated with phytohormones, such as gibberellin, auxin, and abscisic acid.

The role of miRNAs in MYB transcription factor expression in cotton RNAi lines have been reported [57]. Many target genes for these miRNAs have been predicted. For example, cytochrome P450-like TATA box binding protein (TBP) was targeted by nine novel miRNAs [57]. The importance of artificial miRNAs (amiRNAs) and short synthetic interfering siRNA oligonucleotides in RNA interference of cotton has been reviewed earlier [58]. Many aspects of cotton miRNAs and sRNAs for the RNAi process were also discussed in great length elsewhere [59].

8. Conclusions

This cogent review of past and current investigations of cotton miRNAs and sRNAs underscores their vital and expanding importance in regulating growth and developmental processes. This includes plant architecture, somatic embryogenesis, ovule and fiber development, and fertility, as well as resistance to biotic and abiotic stresses through efficient and orchestrated regulation of gene expression. This review highlighted the regulatory role of miRNAs/sRNAs in governing complex traits in cotton, especially fiber initiation, fiber growth and development, and in secondary wall growth and fiber senescence.

However, in this dynamic field, nothing is static and additional studies will be needed on miRNAs and sRNAs for the fine-tuning of targeted traits using RNA interference by employing artificial miRNA vectors and synthetic oligonucleotide duplex vectors. Future investigations will also aid in elucidating the role of these tiny RNAs in epigenomic inheritance associated with agronomically important traits. Genetic engineering methods will aid in monitoring the gene regulatory mechanisms including genomic and epigenomic interactions that control multiple genes or agronomically important traits [58, 59]. Collectively such transgenomic technologies in cotton improvement will help in developing better cultivars that can withstand adverse and disease conditions to benefit the cotton growers and breeders in the world.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Author contribution

MA, IA– coordinated, wrote, and revised manuscript; MM - contributed to writing the manuscript; ZB, KU, DU and RN - collected and analyzed literature, prepared tables, and drafted subsections; VS, CE, SK and IA – critically read, revised, and edited manuscript.

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