Journal of Advances in Biology & Biotechnology

22(4): 1-17, 2019; Article no.JABB.53855
ISSN: 2394-1081

Salient Genetic Notes on Small-RNAs and their Applications in Agricultural Biotechnology

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/JABB/2019/v22i430131
Editor(s):
(1) Dr. Joana Chiang, Department of Medical Laboratory Science and Biotechnology, China Medical University, Taiwan.
Reviewers:
(1) J. Amudha, ICAR-Central Institute for Cotton Research, India.
(2) Suoyi Han, Henan Academy of Agricultural Sciences, China.
(3) Zewei An, Chinese Academy of Tropical Agricultural Sciences, China.
Complete Peer review History: http://www.sdiarticle4.com/review-history/53855

Review Article

Received 01 November 2019
Accepted 07 January 2020
Published 30 January 2020

ABSTRACT

Small-RNAs are 20 to 27 nucleotides long non-protein-coding RNAs that act on either DNA or RNA to effect the regulation of gene expression. Small-RNAs are key in genetic and epigenetic regulation of diverse biological processes and pathways in response to biotic and abiotic environmental stresses. The gene regulatory functions of small-RNA molecules enhance healthy plant growth and normal development by controlling biological processes such as flowering programming, fruit development, disease and pests resistance. Small-RNAs comprise mainly microRNA and small interfering RNA species. MicroRNAs have been proven to primarily engage in posttranscriptional gene regulation while small interfering RNA have been implicated mainly in transcriptional gene regulation. This review covers the recent advancements in small-RNA research in plants, with emphasis on particularly microRNAs and small interfering RNA biogenesis, biological functions and their relevance in the regulation of traits of agronomic importance in plants. Also discussed extensively is the potential biotechnological applications of these small-RNAs for crop improvement.

Keywords: Genetics; small RNAs; microRNAs; agriculture; biotechnology; gene silencing.

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1. INTRODUCTION

Small-RNAs refer collectively to different classes of 20 to 27-nucleotide long non-protein-coding RNAs that act on either DNA or RNA to effect the regulation of gene expression. Small-RNAs mediate gene regulation by cleavage of cognate messenger RNAs, translational repression and transcriptional control through DNA and/or histone methylation [1]. These molecules enhance efficient plant growth and development by regulating processes involved in genome stability, and adaptive responses to biotic and abiotic stresses [2]. Two classes of small-RNAs namely, microRNAs (miRNAs) and small interfering RNAs (SiRNAs), have been identified to play important roles in eukaryotic development. In plants, microRNAs were first unveiled in *Arabidopsis thaliana* in 2002 [3].

This landmark discovery opened a new era in plant post-transcriptional gene regulation by microRNAs. Subsequently, hundreds of microRNAs have been found in diverse organisms [4]. These molecules are not conserved between animals and plants but they are characteristically highly conserved within each kingdom. Notably, there has been rapid discovery of several conserved and non-conserved microRNAs through cloning and deep sequencing of small-RNAs and transcriptome libraries of several plant species including *A. thaliana* [5]; *Oryza sativa* (rice) [6]; *Lycopersicon esculentum* (tomato) [7]; *Triticum aestivum* (wheat) [8]; *Manihot esculenta* (Cassava) and sexual and apomictic *Boechera* [9,10]. Today, major databases including the plant microRNA database (PMRD, *http://bioinformatics.cau.edu.cn/PMRD*) and the miRBase (*http://www.mirbase.org/*) have been established that catalogue discovered microRNAs across diverse organisms.

Small interfering RNAs are chemically similar in nature to microRNAs. They are both derived from double stranded RNAs (dsRNAs), processed into 21-22 nucleotide single stranded molecules by Dicer or a Dicer-like (DCL) enzyme, and subsequently incorporated into a RNA-induced silencing complex to guide the cleavage or translational repression of the complementary strand [11]. Functionally, Small interfering RNAs and microRNAs are equivalent in their regulation of gene expression, and participate in partially overlapping pathways. Nonetheless, substantial differences occur in their biogenesis and regulatory functions. Generally Small interfering RNAs arise from aberrant dsRNAs or from exogenous agents such as viruses, and silence the same molecule from which they originated. Small interfering RNAs are processed from perfect or near perfect RNA duplexes [12]. In contrast, microRNAs are derived from nucleolytic processing of microRNA gene transcripts that form extensive but imperfect stem-loop structures. MicroRNAs act *in trans*, silencing messenger RNAs from other genes. SiRNAs are categorized into transacting siRNAs (tasiRNAs), natural antisense siRNAs (natsiRNAs) and repeat associated siRNAs (rasiRNAs).

A better understanding of the basic concepts of small RNA biology will enable researchers to fashion out novel approaches aimed at efficient plant production and utilization. Several excellent reviews have described the characteristics, biogenesis, and functional mechanisms of plant small RNAs [13,14]. This overview has attempted to present a simplified updated plant small RNAs research, highlights their biogenesis, multifunctional roles in plant growth and development and potential biotechnological applications.

2. MICRORNAS (MIRNA) BIOGENESIS IN PLANTS

The biogenesis of microRNAs involves initially RNA polymerase activities followed by series of nucleolytic cleavage processing in the nucleus and the cytoplasm [15]. MicroRNAs formation commences with RNA polymerase II controlled transcription of a microRNA gene (*MIR* gene) in the nucleus into primary RNA transcripts with imperfect self-complementary fold-back regions referred to as pri-microRNAs. These transcriptional units are subsequently capped at the 5' end, while the 3' end is polyadenylated [16]. The pri-microRNAs is further processed by an enzyme microprocessor complex that includes RNase type-III endonuclease Dicer Like-1 (DCL1) and the *Arabidopsis* hyponastic leaves (HYL1), a dsRNA-binding domain containing protein, bound to the pri-microRNA complex [17]. The activity of the microprocessor culminates in microRNA precursor stem-loop structures, pre-microRNAs from which duplex microRNAs are further excised and processed [18] (Fig. 1).

DCL1 processes the pre-microRNAs into a miRNA-miRNA* duplex with two nucleotide overhangs at the 3' end [3]. HYL1 and Serrate, a zinc finger protein, assist DCL1 in releasing the

microRNA duplex [20]. Next, the Hua Enhancer1 (HEN1), a methyl transferase, adds methyl groups to the 3' ends of the microRNA duplex and stabilizes it [21] (Fig. 1). The microRNA duplex is then exported into the cytoplasm by HASTY (HST), a plant ortholog of a microRNA transporter enzyme, Exportin-5, which in animal systems transports pre-microRNAs to the cytoplasm [22]. Based on the relative internal thermodynamic stability of the two ends of the duplex, one strand becomes the mature microRNA whereas the other complementary strand is degraded by an unknown nuclease [23]. The mature microRNA is incorporated into an RNA-Induced Silencing Complex (RISC) mediated by Argonaute proteins (AGO) in order to repress translation or direct cleavage of target messenger RNAs [24]. In plants, microRNA-mediated regulation is assumed to occur almost exclusively by cleavage, while the contribution of translational repression is considered insignificant. Genome-wide transcriptome analyses have revealed very low levels of translational repression in target genes in plants [25].

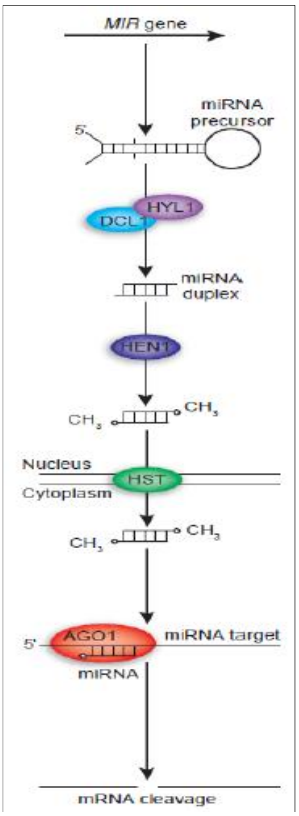

Fig. 1. A microRNA (miRNA) biogenesis pathway in plants (Figure adopted from Mallory and Vaucheret, [19])

3. FORMATION OF SMALL INTERFERING RNAS (SiRNAs)

3.1 Transacting siRNAs (tasiRNAs)

Trans-acting siRNAs in plants are 21-nucleotide RNAs that are encoded in intergenic regions. They act *in trans* on messenger RNAs to induce cleavage. Trans-acting siRNAs are derived from double-stranded RNA produced by RNA-Dependent RNA Polymerase 6 (RDR6) [26,27]. During transacting siRNAs biogenesis (Fig. 2), secondary siRNAs are produced in order to amplify the effect of RNA silencing. Production of secondary siRNAs is triggered by the interaction of microRNAs or small interfering RNAs siRNAs with a long target RNAs. A number of the important auxin responsive transcription factors (ARF3, ARF4) have been shown to be tasiRNA targets. Other known tasiRNA targets include genes encoding the pentatricopeptide repeat family proteins and putative MYB transcription factors [28,29].

3.2 Natural Antisense siRNAs (Natsi RNAs)

Natural antisense siRNAs originate from dsRNA precursors formed by transcription of two partially overlapping genes that harbour regions of complementarity at their 3' ends [33]. The size of natural antisense siRNAs molecules range from 21 to 24 nucleotides and they originate from overlapping sense and antisense transcripts (Fig. 3). Natural antisense siRNAs are important in several developmental and response mechanisms in plants, such as pathogen resistance, salt tolerance and cell wall biosynthesis. Studies on plant response to high salinity indicate that the natsiRNA pathway plays some function in plant adaptive protection mechanism in response to either abiotic or biotic stress. Katiyar-Agarwal et al. [34] also revealed that pathogen-induced natsiRNAs in bacteria enhance the host defense response by repressing a putative negative regulator of the disease resistance pathway.

3.3 Repeat Associated siRNAs (rasi RNAs)

In *A. thaliana*, repeat sequences are an extremely prolific unique class of siRNAs. The repeat associated siRNAs (RasiRNAs), are 24 nucleotides long. RasiRNAs are involved in transcriptional gene silencing of repetitive DNA sequences in plant genomes by direct DNA methylation [36] (Fig. 4). The RasiRNAs pathway became important due to two main

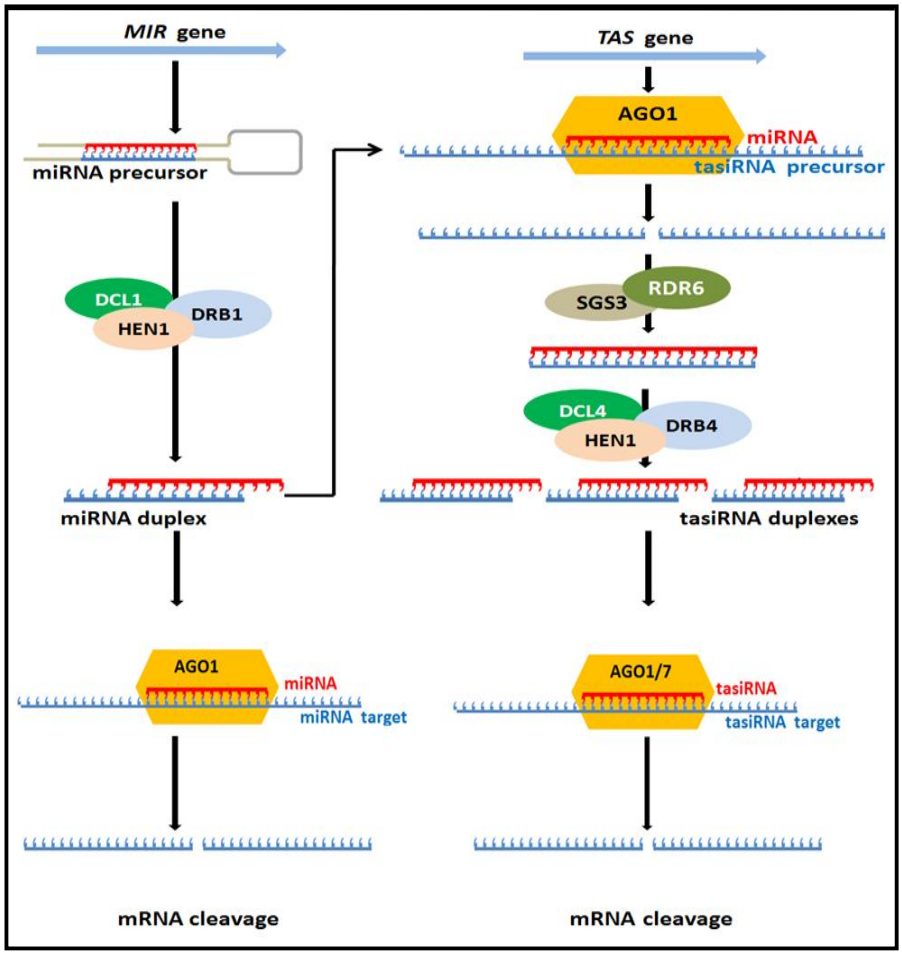

Fig. 2. The transacting small interfering RNAs (tasiRNAs) mechanism integrates both the microRNA and siRNA biogenesis pathways. The expression of tasiRNAs is initiated with transcription of MIR genes, followed by processing into a mature microRNA to yield tasiRNA transcripts that contain microRNA target site(s). These mature microRNA targets their transcripts and guide the cleavage of the transcript in a miRNA/DCL1/HYL1-mediated sequence dependent fashion. Notably, instead of being silenced, the cleaved transcripts are used as templates to synthesize dsRNA, a process that also requires Supressor of Gene Silencing 3 (SGS3) [30]. Subsequently, a series of sequential cleavages of the dsRNA by DCL4 [31], result in a phased array of 21-nt tasiRNAs. These tasiRNA molecules are then incorporated into AGO7- containing RISCs, and negatively regulate gene expression via direct cleavage of their own specific cognate mRNAs for degradation [32] (Figure modified from Mallory and Vaucheret, [19])

pioneering works. Wassenegger et al. [37] were the first to demonstrate the possibility for homologous transgenes to be methylated in RNA viroids. Later, Jones et al. [38] further showed that nuclear DNA could be methylated by introducing a homologous cytoplasmically replicating RNA virus. Both groups speculated that a sequence-specific RNA signal was able to enter the nucleus to direct DNA methylation.

4. PROTEIN INTERACTIONS IN SMALL RNA BIOGENESIS

4.1 DCL and HYL Mediated Pri- and Pre-microRNA Processing

In plants, a ribonuclease III-like protein in the nucleus, Dicer-Like 1 (DCL1), equipped with two tandem N-terminal dsRNA binding domains

(dsRBDs), is responsible for catalyzing both pri-microRNA and pre-microRNA processing [40]. Across diverse plant species, a huge population of conserved and non-conserved small-RNAs processed as DCL1-dependent 21 and 24 nucleotide molecules occur. In *A. thaliana* the longer microRNAs are predominantly produced in floral structures where it has been observed that DCL3, the synthesizing enzyme, is many folds more abundant than in leaves. Based on this finding Vazquez et al. [41] speculated that the organ-specific competition between DCL1 and DCL3 might constitute regulatory mechanism controlling the production of organ-specific microRNA molecules. The Hyponastic Leaves1 (HYL1) protein, also has two dsRBDs [42], a putative nuclear localization site, and a putative protein–protein interaction domain. HYL1 has been implicated in microRNA accumulation and may play the same role as Pasha/DGCR8 for DCL1 in plants [43]. DCL1 forms a complex with HYL1 *in vitro* and *in vivo*.

The plant-specific zinc finger protein, Serrate, is also known to interact with DCL1 during nuclear processing of microRNAs [20]. HYL1 binds to RNA substrates in association with DCL1, to promote the accurate processing of microRNA precursors [43]. Yu et al. [44] observed low accumulation of DCL1 and HYL1, as well as poor pri-microRNA accumulation, in pleiotropic *A. thaliana* Dawdle (DDL) mutant, despite unaltered MIR gene transcription. It is likely that the DDL encoded nuclear RNA-binding protein stabilizes pri-microRNAs by interacting directly with DCL1. It is also known that stem loops of SINE elements mimic the hairpin structures of microRNA precursors and bind to HYL1 [45]. Thus, specific expression of SINE RNAs might affect the balance of HYL1 influence on the production of plant microRNAs.

4.2 DICER-Like and HEN Mediated Small RNAs Maturation

In *A. thaliana*, dsRNA is processed into specifically sized small RNA duplexes by one of four Dicer-like (DCL1-4) proteins. Two of these DCL proteins are likely localized in the nucleus [46]. DCL1 possibly performs both Drosha and Dicer-like activities for microRNA maturation inside the nucleus. DCL1 mainly produces 18-21 nucleotides (nts) small RNAs. In contrast, the products of DCL2, DCL3, and DCL4 are 22, 24 and 21-nts, respectively. In *O. sativa*, DCL3 also processes 24 nts microRNA-like siRNA from multiple microRNA fold-backs [47]. These small RNAs are predominantly DCL3-dependent, but some loci require initial processing by DCL1, giving rise to both 21- and 24-nts small RNAs. The 24-nucleotide microRNA-like small interfering RNA fraction preferentially associates with *O. sativa* AGO4 variants, and guide methylation of target DNA [47]. Chellappan et al. [48] showed that the 24-nucleotide small RNA molecules are not microRNAs, but rather microRNA-related small interfering RNAs generated by DCL3/RDR2/Pol IV through the heterochromatin small RNA interfering pathway. This is supported by the fact that accumulation of the 21-nucleotide small RNA species of miR2883 and miR2328 was unaffected in RDR2, RDR6 and NRPD1 mutants, but drastically reduced in DCL1, HEN1 and HYL1 mutants.

On the other hand, the 24-nucleotide small RNA species lacked expression in DCL3 as well as RDR2 mutants [48]. The 24-nucleotide siRNAs are known to be heterochromatin-associated, and are often referred to as *cis*-acting siRNAs because they affect the genomic loci that produce them, often resulting in their transcriptional gene silencing. Studies have further identified a novel population of longer microRNAs (23–27 nts) generated by DCL3, RDR2 and Pol IV from a typical heterochromatic siRNA biogenesis pathway in *A. thaliana*, *O. sativa* and *Physcomitrella patens* [48]. These longer microRNAs are specifically associated with the AGO4 protein.

Plant microRNAs comprise a heterogeneous collection of hairpins with variable size and shape [49]. However, little information about the structural requirements for microRNA processing is available in plants [50]. The current established canonical model indicates that plant pri-microRNAs are first cleaved by DCL1 to release their fold-back precursors, which are then further processed by DCL1 to generate the microRNA molecules [40]. The RNA structural determinants that dictate how a microRNA is excised in plants are still not clear, although it appears that the first loop-distal cut is often at a point of an imperfectly base-paired region of approximately 15 nucleotide between the miRNA-miRNA* duplex, and either a less structured region of the lower stem or its loop end [51].

Mature microRNA duplexes are stabilized by the S-adenosyl methionine- dependent methyl transferase HEN1, which methylates all

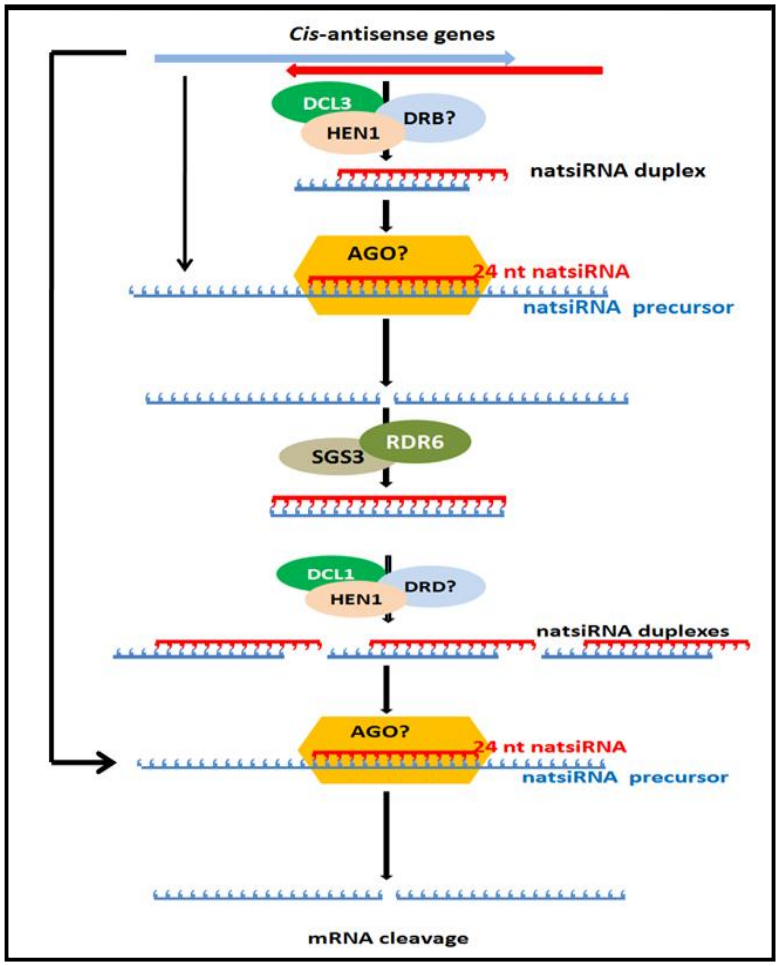

Fig. 3. DsRNAs formed by the *cis*-antisense transcripts provide a substrate for either DCL1 or DCL2 cleavage activity, and are processed into single primary 24-nucleotide natsiRNA species. A single 24- nucleotide natsiRNA subsequently targets one of the *cis*-antisense gene pair transcripts which are constitutively expressed for cleavage. The induced expression of the other genes therefore governs the formation of the dsRNA. The cleaved RNA transcript molecule is converted into dsRNA in a RDR6- and SGS3-dependent manner. The RDR6/SGS3-synthesized dsRNA molecule is then processed into a phased array of secondary 21-nucleotide natsiRNA species by DCL1. The phased 21-nucleotide natsiRNAs, further promote the silencing of the homologous mRNA transcripts [35] (Figure modified from Mallory and Vaucheret, [19])

plant-silencing small RNAs. Methyl groups deposited on the 3' terminal nucleotides of each strand prevent their uridylation and subsequent degradation [52]. The isolation of a small-RNA degrading nuclease *in vitro* in *A. thaliana* further underscores the importance of microRNA stability control in plants [53]. Besides, simultaneous knockdown of three members of the Small-RNA Degrading Nuclease gene family have been found to elevate microRNA levels and cause developmental defects in *A. thaliana.*

4.3 ARGONAUTEs: RNA-induced Silencing Complex Proteins

Small-RNA duplexes released upon dicing are either retained in the nucleus for chromatin-level activities or exported to the cytoplasm by the HASTY protein for posttranscriptional gene silencing. A selected small RNA strand is incorporated into one or several RNA-induced silencing complexes (RISCs) that scan the cell for complementary nucleic acids to execute their function. The core component of the RISC

machinery is the Argonaute (AGO) protein family, which have a small RNA-binding PAZ and PIWI domains with catalytic residues conferring endonucleolytic activity for slicing complementary RNAs [54]. Argonaute effectors mediate messenger RNA cleavage, translational repression, or epigenetic DNA modification. The main activities of RISC include RNA endonucleolytic cleavage or ‘‘slicing’’ of small RNA-target hybrids, repression of gene expression via translation inhibition and DNA cytosine and/or histone methylation [46].

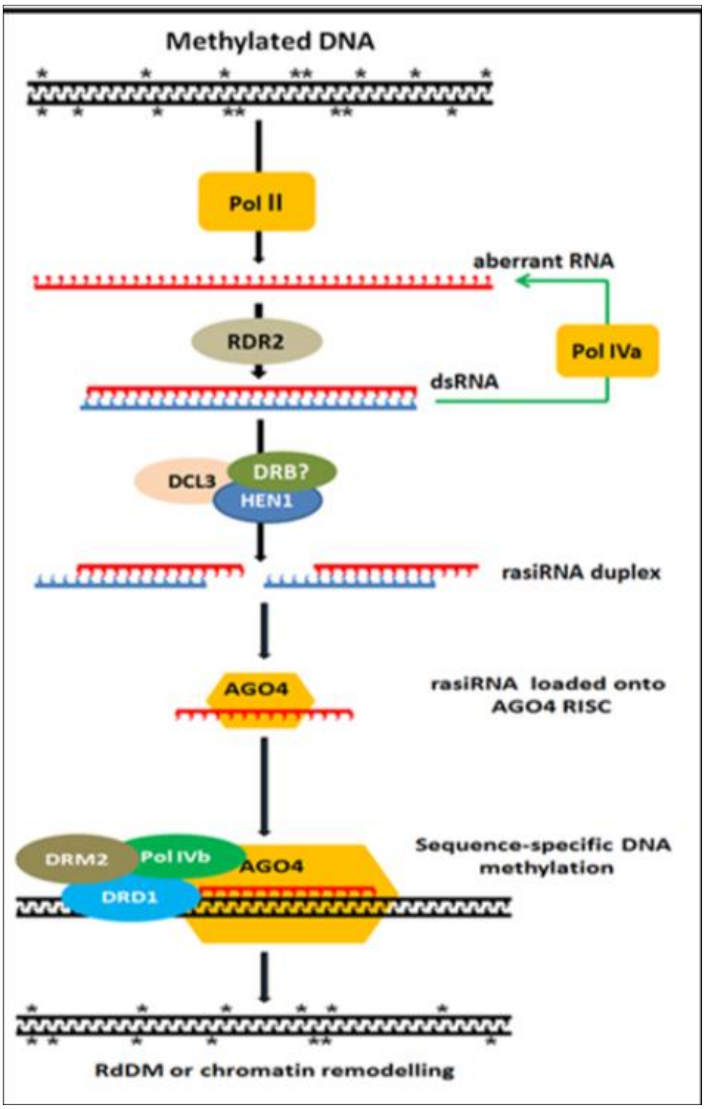

Fig. 4. Methylated DNA acts as a template for the transcription of aberrant RNAs. The transcribed aberrant RNA is then converted to a double- stranded RNA by RNA dependent RNA polymerase, RDR2. DsRNA is used as template to produce additional single stranded aberrant RNA molecules which again are converted to dsRNA by RDR2 to form a self-perpetuating rasiRNAs signal amplification loop [30,39]. The resultant dsRNA is cleaved by DCL3 into 24-nucleotide rasiRNAs and methylated by HEN1. The processed rasiRNAs are loaded onto AGO4 RISC, which directs the sequence specific DNA methylation step of RdDM by the combined action of the methyl transferases, Defective in RNA-directed DNA methylation1 (DRD1) and Domains Rearranged Methylase 2 (DRM2) (Figure modified from Mallory and Vaucheret, [19])

During plant microRNA-guided silencing, central mismatches in the microRNA-target messenger RNA duplex lead to translational repression through slicing inhibition, while perfect central matches in the microRNA-target messenger RNA duplex tend to cleave target messenger RNA and exclude translational repression regardless of a few mismatches in other regions [24]. MicroRNA directed cleavage occurs more frequently in the center of microRNA-target complex, between the residues that pair with nucleotides 9–11 of the guiding microRNA, suggesting a ‘‘slicing’’ mode of action which resembles siRNA-directed silencing. The cleavage target sites seem to be commonly located in coding regions in plants. There are several RNA silencing pathways that are variations of the basic mechanism of plant microRNA functions, but all culminate generally in the repression of targets, triggering of siRNAs from targets, and triggering of RNA-directed DNA methylation ([54]; Fig. 5).

In *A. thaliana* there is a ten member multigene family for Argonaute proteins involved in microRNA biogenesis and function. Furthermore, small RNA-target node identity or functionality may be modulated at the transcriptional, AGO sorting, AGO activity and intercellular transport levels [54]. Among the Argonaute members, AGO1, AGO4, AGO6 and AGO7 are involved in small RNA-directed silencing, whereas ‘‘slicer’’ activity has been proven for both AGO1 and AGO4 [55].

Data presented by Mi et al. [56] indicate that AGO1 is loaded with most microRNA and tasiRNA by selective recognition of a 5' uridine (5'U), leading to microRNA target cleavage. This is consistent with evidence revealed by hypomorphic *AGO1–27* mutants where near-normal levels of microRNA target transcripts are generated, yet levels of protein from these transcripts were found to be disproportionately high [57]. In the *AGO1–27* mutants the slicing activity is inactivated by a point mutation which prevents translational repression by disrupting interactions between AGO1 and other proteins specifically required for translation repression [58]. A high frequency of 5’U occurs in the vast majority of microRNAs and tasiRNAs and has been attributed to the selective binding of a 5' uridine (5'U) microRNAs by AGO1.

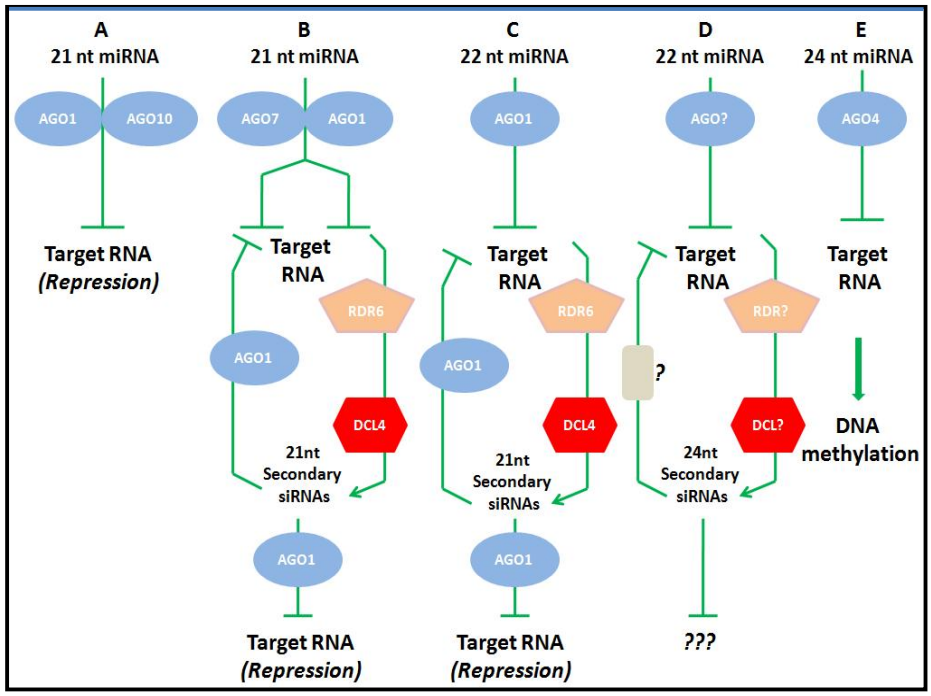

Fig. 5. Functional network of plant microRNA pathways. The general mechanism of plant microRNAs functions include mainly repression of targets, triggering of siRNAs from targets, and triggering of RNA-directed DNA methylation. Pathways A, B, and E have been described in monocots, eudicots and bryophytes, pathway C in monocots and eudicots, and pathway D only in monocots (Figure modified from Cuperus et al., [14])

However, there are a few notable exceptions, such as miR172, miR169 and miR395 isoforms, which possess either 5'A or 5'C but still associate with AGO1. AGO2 and AGO5 have clear preferences for small-RNAs with 5'A and 5'C, respectively [56]. Similarly, AGO4, which acts with 24-nucleotide siRNAs to specify RNA-directed DNA methylation, has an apparent preference for 5'A. In contrast, AGO7 has high specificity for the small-RNA miR390.

5. SMALL RNA INTERFERENCE GENE SILENCING

RNA silencing via RNA interference (RNAi) involves RNA-guided regulation of gene expression in which dsRNA inhibits the expression of genes with complementary nucleotide sequences. RNAi is a conserved regulatory process that is a vital part of the plant protection immune response which allows the entire plant to prevent or minimize the effect of viruses and other pathogens after an initial localized encounter [59]. The RNA-silencing mechanism encompasses four main processes: induction by dsRNA, dsRNA processing into 20–27 nucleotide small RNAs, 3'-methylation of small RNA and small RNA incorporation into Argonaute effector protein complexes that associate with partially or fully complementary target RNA or DNA [60]. DsRNA might arise from transcription of inverted-repeat sequences and virus replication, or can be generated at loci that produce transcripts with internal stem-loop structures. They could also be synthesized by either one of the six members of RNA-dependent RNA polymerases (RDR1–6) that copy single-stranded RNA [61]. DsRNA suppress specific transcripts in a sequence-dependent manner.

RNAi mediated gene silencing occurs via dsRNA with complementary nucleotide sequences. These dsRNAs guide transcript degradation or inhibition of protein synthesis for the regulation of gene expression, control of development, or cell defense against invading nucleic acid sources (e.g. viruses, transposons or transgenes). The RNA silencing mechanisms namely transacting siRNAs (tasiRNAs), natural siRNA (natsiRNA) and repeat associated siRNA (rasiRNA) use diverse small interfering RNA classes to recognize and manipulate complementary nucleic acids. Most microRNAs target mainly transcription factors and other important genes involved in almost all aspects of plant growth and development [62].

6. MICRORNAS IN PLANT GROWTH AND DEVELOPMENT

6.1 Leaf Morphogenesis, Developmental Timing and Patterning

MiR390 and miR319 are involved in the leaf development, including morphogenesis, growth polarity via regulating their target genes TAS3-ARF, TCP and GRF. MiR319 regulates leaf morphogenesis by controlling levels of TCP family of transcription factors [63]. Mutation in the miR319 binding sites of Lanceolate, a member of the TCP transcription family, resulted in small simple leaves instead of normal compound leaves in tomato [64]. On the contrary, over expression of miR319 in transgenic tomato plants produced larger leaflets and continuous growth of leaf margins, indicating miR319 mediated regulation of leaf shape.

In *A. thaliana,* miR390 regulates target genes indirectly through production of trans-acting small interfering RNAs locus [65]. These trans-acting small interfering RNAs negatively regulate ARF3 and ARF4, which are necessary for proper leaf development, leaf polarity, developmental timing and patterning [29]. A number of genes in auxin signaling have been identified and confirmed as targets of microRNAs. One such gene, the TIR1 auxin receptor, is a predicted target of miR393 [66].

6.2 Flowering Time Regulation and Organ Development

MiR156 and miR157 negatively regulates messenger RNAs of transcription factor genes family known as Squamosa Promoter Binding Protein-Like (SPL) [9,67]. These transcription factors are involved in developmental timing in *A. thaliana*. Plants expressing miR156 and miR157 resistant forms of SPL3/4 and SPL5 flower earlier whereas constitutive overexpression of miR156 results in a prolonged vegetative phase and late flowering [68]. In tomato, the Colourless Non-Ripening (CNR), an SPL transcription factor member, is targeted by miR156 and miR157. Manning et al. [69] reported that CNR mutants have inhibited normal fruit ripening and exhibit colorless fruit lacking cell-to-cell adhesion. Furthermore, Amiteye et al. [10] found that SPL11 is differentially expressed in ovules of sex and apomictic *Boechera* species. Although SPL11 does not seem to be a key factor in apomixis, it might be associated with DNA sequence variation in regulatory factors among apomictic hybrid species.

The miR159 family regulates two MYB domain transcription factor genes, MYB33 and MYB65, and the TCP (Teosinte Branched1, Cycloidea, and PCF) family gene transcripts in floral organ development [70]. MYB proteins bind to promoters of a number of genes, including the floral meristem identity gene LEAFY [71]. Millar and Gubler [72] reported that transgenic plants over-expressing a miR159-resistant version of MYB33 show aberrant phenotypes, delayed transitions to flowering under short day conditions, and infertility due to defective anther development, suggesting that down-regulation of MYB33 by miR159 is important for normal organ development. A report in *A. thaliana* suggests that miR159a and miR159b are functionally redundant in controlling plant growth habit, leaf, silique, and seed development [73]. It has also been shown that the plant hormone ABA has a regulatory role on the levels of miR159 during seed germination. MiR159 accumulates in response to ABA during seed germination, resulting in the degradation of its target mRNAs (MYB33 and MYB101) to desensitize hormone signaling during seedling stress in *A. thaliana* [74].

Both microRNAs miR165 and miR166 target HD-Zip transcription factor family members [70] such as Phabulosa (PHB), Phavoluta (PHV) and Revoluta (REV), which are involved in leaf and vascular development and leaf polarity [75]. Dominant gain-of-function mutations in PHB, PHV and REV genes result in adaxialized leaves and floral organs. The HD-zip genes are expressed in the meristem and in the adaxial domain of lateral organs. It has also been reported that miR165 is involved in HD-ZIP-III mediated indeterminacy in apical and vascular meristems [76]. MiR165 and miR166 also cause DNA methylation of the PHB and PHV genes, and likely induces transcriptional silencing of these genes in the abaxial domain [77].

Another microRNA gene, miR172 controls the flowering time and floral organ pattern in *A. thaliana* via down-regulation of the Apetalata2 (AP2) like transcription factor genes [78]. MiR172 is important for the floral transition in many plants, including tomato, apple and so on. The AP2 family belongs to class A genes of floral organ identities. Transgenic plants expressing *A. thaliana* wild type AP2 and miR172 resistant AP2 genes did not show any phenotype due to regulation by the endogenous miR172*,* rather the overexpression of a miR172-resistant form of AP2 cDNA showed floral patterning defects, indicating the loss of floral organ determinancy [79]. Other AP2-like genes, such as Target of Eat1 (TOE1), TOE2 and TOE3 in *A. Thaliana,* or Indeterminate Spikelet1 and Gloy15 in *Z. Mays,* are also under the regulation of miR172. Over-expression of TOE1 delayed flowering in *A. thaliana* whereas mutants over-expressing miR172 were early flowering. Gloy15 is a transcription factor involved in the development of adult leaf traits in *Z. mays*. All these observations suggest a crucial role of miR172 in repressing AP2 genes in the identity of floral organs and flowering time. Chuck et al. [80] also found that the two microRNAs play antagonistic roles in flowering induction. MiR172 and miR156 levels are complementary during juvenile to adult shoot development, indicating that the relative levels of these two microRNAs might determine the developmental phase transition. High level of miR156 extends juvenile phase and delays flowering, while miR172 accumulation leads to early flowering.

6.3 Fruit Initiation, Size Formation and Fruit Ripening

Many conserved microRNAs have been implicated in fruit development and have been confirmed to participate in diverse aspects of the physiology of fruit development, for instance fruit initiation, size formation, coloration, and ripening [81]. Several Auxin Response Factors (ARFs) such as ARF6, ARF8, ARF10, ARF16 and ARF17 contain microRNA binding sites. These factors play very important roles in auxin mediated growth and developmental responses. MiR160 guides the cleavage of ARF10, ARF16 and ARF17 mRNAs [82]. Mallory et al. [83] observed that expression of miR160 resistant ARF17 resulted in the altered expression of auxin-responsive genes, which led to severe pleiotropic abnormalities, including leaf shape, development of premature inflorescences, sterile and abnormal stamens, decreased petal size and defects in root growth. MiR160 is also involved in root cap formation by controlling root cap cell differentiation [84]. These data indicate that miR160-mediated regulation of the ARFs is critical for healthy plant growth and development. Furthermore, miR160 might play an important role in maintaining proper auxin signaling homeostasis.

MiR164 negatively regulates messenger RNAs of genes that encode for the transcription factors Cup-Shaped Cotyledon1 (CUC1) and CUC2 [70]. These genes are necessary for the formation of boundaries between meristem and emerging organ primordia [85]. CUC1 and CUC2 mRNAs

are normally restricted to the regions between developing petal and sepal primordia. Expression of a miR164-resistant form of CUC1 in wild type plants resulted in reduced sepal number, increased petal number, and broadened leaves [86,87]. With an inducible expression system, it was found that expression of miR164-resistant CUC2 led to an increase in the width of the boundary domain between sepals. Expression of miR164-resistant CUC2 could restore sepal separation to miR164 overexpressing lines [88]. In CUC1 and CUC2 double-mutant seedlings of *A. thaliana,* the two cotyledons failed to separate and the seedling meristem was arrested [89]. All results emphasize the importance of miR164 mediated regulation for proper organ formation [90].

Four members of the MIR167 family in *A. thaliana* exhibit specific functionality in the expression of distinct floral organ domains that correspond to organ specific function of their targets, ARF6 and ARF8 [91]. Wu et al. [92] observed that while all four MIR167 family members coordinate to regulate targets, ARF6 and ARF8 mRNAs are targets of miR167. The miR167c-ARF8 and miR167b-ARF8 complexes function uniquely in anther filaments and ovules respectively. MiR168 regulates the expression of the *A. thaliana* AGO1 protein, which is essential in overall microRNA functions due to the association of a majority of 21 nucleotide long small RNAs with the AGO1-RISC complex [93]. MiR168 regulates its own pathway component, AGO1, through an auto-regulatory mechanism to maintain homeostasis of AGO1 for proper development. Since it regulates the key component of RISC, any variation in this microRNAs expression has potential influence on the function of other microRNAs.

A family of putative transcription factors known as Scarecrow-Like Proteins [70], are involved in radial patterning of roots and hormone signaling [94]. The efficient function of these genes in plant development is under the control of miR171. Functional characterization analyses by Llave et al. [95] in *A. thaliana* and *Nicotiana benthamiana* revealed a relatively high level of miR171 in the inflorescence and flower tissues compared to stem and leaf.

6.4 Anthocyanin Biosynthesis, Lignification and Fruit Soluble Solids

MiR828 and miR858, play versatile roles directly or indirectly in anthocyanin biosynthesis. MiR828 and miR858 co-regulate by repressing genes encoding MYB transcription factors and control the biosynthesis of anthocyanin in plants [96]. MiR828 intensifies silencing effect via secondary phasiRNAs production from targeted MYB genes [97]. In Arabidopsis, overexpression of miR828 reduces anthocyanin accumulation [98]. In tomato, miR858 similarly exhibits a repressive effect on anthocyanin biosynthesis. The silencing of MIR858 results in increased anthocyanin content by modulating the expression of SlMYB7 and SlMYB48 [99].

Another trait of important consideration is fruit lignification which is known to be controlled by laccases. MiR397 has been shown to contribute to enhancing fruit quality in pear by inhibiting expression of the laccase gene [100]. A single nucleotide polymorphism (SNP)—associated with low levels of fruit lignin—in the MIR397 gene promoter has been reported. This SNP could have practical application in marker assisted selection for low lignin content in fruits. Another important trait generally desired in fruits is high soluble solids content [101]. It has been discovered in different strawberry cultivars that high content of soluble solids is positively correlated with high level of Pi content. The regulation of Phosphorus nutrition by microRNAs is well established. MiR399 has been reported to respond to Pi-starvation by guiding the cleavage of PHO2 RNA, which encodes an E2 ubiquitin conjugase-related protein that negatively affects Pi content and remobilization [102]. Overexpression of miR399 can significantly improve fruit quality by increasing the Pi content and thereby the soluble solid content in strawberry fruit.

6.5 Viral, Bacterial and Fungal Disease Resistance

MiR398, miR482 and miR528 are very important in plant viral, bacterial or fungal disease resistance. MiR398 and miR528 mediate disease resistance by targeting a group of oxidases, including laccase, ascorbic acid oxidase, superoxide dismutase. These oxidases have been proven to enhance plant defense through the regulation of the level of reactive oxygen species. MiR482 on the other hand, mediates disease resistance by regulating many of the NB-LRR resistance genes. In tomato infested with viruses or bacteria, miR482 was found to be down-regulated while some of its disease-resistant NBS-LRR target genes are up-regulated [103]. Similarly, in cotton seedlings infected with *Verticillium dahlia*, a fungal

pathogen, miR482 family genes were detected to be suppressed leading to the induction of the expression of specific NBS-LRR genes to activate disease defense [104].

7. SMALL RNAS IN AGRICULTURAL BIOTECHNOLOGY

7.1 Plant Protection against Disease Agents

Significant yield losses due to attacks by parasitic nematodes, herbivorous insects, parasitic weeds, and fungi, occur globally in important plant species [105]. One of the many strategies employed to mitigate the effects of these pathogenic agents is RNA interference (RNAi), where the expression of dsRNA is directed against suitable eukaryotic pathogen target genes in transgenic plants. This has been shown to give protection against harmful eukaryotic species [106]. Plant genomes express siRNAs in response to infection by specific types of parasites. In *A. thaliana* multiple dicer homologs with specialized functions are expressed against different types of viruses and bacteria [107]. These genetic activities may be part of a generalized response to pathogens that down-regulates any metabolic processes in the host that aid the infection process.

The RNAi defense mechanism can silence a gene throughout an organism or in specific tissues. SiRNA mediated silencing signals in plants are not cell autonomous. Spontaneously or artificially triggered silencing signals spread systematically from cell to cell via the plasmodesmata, and through vascular tissues over long distances [108]. Presumably microRNAs are not mobile as free molecules, but are probably transported as complexes in association with particular proteins. It is possible, therefore, for microRNAs to move from a distance source and accumulate within tissues where they may assume stability and effect regulation [109]. Since this technology for generating virus resistance in plants was first demonstrated by Fire et al. [110], the strategy has emerged as a powerful tool for battling some of the most notoriously challenging diseases caused by viruses [105] and bacteria. RNA-mediated functions have greatly increased with the discovery of small non-coding RNAs which play a central part in RNA silencing. The application of tissue-specific or inducible gene silencing, in combination with the use of appropriate promoters to silence several genes simultaneously, will result in crop protection against destructive pathogens. For instance, host-derived RNAi is being applied to develop plant parasitic nematodes resistant crops [106].

On the other hand, many plant pathogens such as viruses respond to defensive mechanisms by evolving elaborate counter mechanisms that suppress RNAi defense in plant cells [111]. Viruses for instance, produce specific proteins that bind short dsRNA fragments with single-stranded overhang ends to subsequently disrupt RNAi defense [34]. Nonetheless, RNAi application has resulted in successful control of many economically important diseases in crops.

7.2 Development of Nutritionally Safe Crop Plants

The stable and heritable nature of plant RNAi phenotypes has been utilized to modify a variety of plants that were hitherto unsafe for human consumption due to naturally high toxin levels [112]. Cotton seeds, though proven to be rich in dietary protein, are unsuitable for human consumption due to their naturally high gossypol content. Gossypol is a toxic terpenoid important in preventing damage to cotton plants by pests. This problem was circumvented by RNAi modified cotton stocks that produce seeds with reduced levels of delta-cadinene synthase, a key enzyme in gossypol biosynthesis. Similarly, the levels of allergens in tomato, the high cyanogenic linamarin content in cassava as well as precursors of likely carcinogens in tobacco plants [113], have been successfully reduced. Other plant products that have been produced via exploitation of the RNAi pathway include fortified tomatoes with dietary antioxidants, the Flavr Savr tomato and ringspot-resistant papaya [114].

8. CONCLUSIONS

Obviously small-RNAs function as key molecules involved in the maintenance of genetic harmony during plant growth and development. This harmony is achieved by various elaborate gene silencing regulatory mechanisms. Better insight into their mode of function will enable more effective biotechnological strategies to enhance plant production.

ACKNOWLEDGEMENTS

The author wishes to profoundly commend the invaluable assistance of many at the Biotechnology Centre that have enabled the successful development of this informative work.

He also acknowledges the Biotechnology and Nuclear Agriculture Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC) for permitting the use of its facilities to carry out this work.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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