Short Interfering RNAs

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Contributor: Koushik Halder , Abira Chaudhuri , Malik Z. Abdin , Asis Datta

RNA interference (RNAi) has inarguably been a revolutionary discovery in the field of biology in the last several decades. The discovery of small (20–30 nucleotide long) non-coding RNAs that can regulate genes and the genome completely transformed RNA biology. These small RNAs can guide effector proteins targeting any complementary nucleotide sequence through the RNAi pathway, thereby downregulating its expression level. Napoli and Jorgensen first reported small RNA-mediated gene regulation in plants while working with chalcone synthase (CHS) in petunia.

small non-coding RNAs siRNA miRNA tasiRNA

1. Biogenesis of Short Interfering RNAs

dsRNA-mediated post-transcriptional gene silencing (PTGS) symbolizes the cellular defense mechanism protecting it from foreign nucleic acids of invading viruses or transposons [1][2]. Such integration of alien genes produces dsRNAs, which act as a guide for sequence-specific RNA degradation and are believed to be associated with maintaining the silencing process for a long time [3][4]. The processing of these long dsRNAs into 21–24 nt long short-interfering RNAs (siRNAs) is facilitated by Dicer, a ribonuclease III enzyme [5] (**Figure 1**A). The presence of these siRNAs was first reported by Hamilton and Baulcombe in plant tissues that showed virus-induced PTGS [6]. These siRNAs were later found to be present also in *Drosophila melanogaster* embryo lysate [7], where the added synthetic 20–22 nt RNA duplexes can efficiently target and cleave mRNAs at 21 nt intervals [8]. That is why these 21 nt long RNAs were called siRNAs or silencing RNAs. Dicer-mediated processing is coupled with other co-factors, and these siRNAs are finally loaded onto the RNA-induced silencing complex (RISC), a member of the argonaute protein family [9]. During incorporation within the RISC, one strand called the “passenger strand” is dissociated by the activity of AGO2, which is encoded by the gene *AGO2* (argonaute RISC catalytic component 2), whereas the other “guide strand” that serves as a guide for RNA-directed sequence-specific silencing stays within the complex, forming the mature RISC [10][11]. Targeted mRNA with sequence complementarity with the 21 nt long guide-siRNA within the mature RISC is cleaved between the 10th and 11th nucleotide (from the 5′ end) by the PIWI domain of the AGO2 protein, generating products containing 5′-monophosphate and 3′-hydroxyl termini [12][13]. These cleaved products are rapidly degraded by the endogenous exonuclease activity due to the lack of 5′ capping or a 3′poly(A) tail [14]. Apart from the above-mentioned RNAi, plants [3][15], fungi [16], and *C. elegans* [4][17] possess a special enzyme called RNA-dependent RNA polymerases (RdRPs), which can produce additional dsRNAs for amplifying the RNAi response. Such dsRNAs are synthesized in a primer-independent manner using the targeted mRNA as a template. It is subsequently processed by Dicer to produce more siRNAs, thereby facilitating the recycling of the RISC complex [18] (**Figure 1**B).

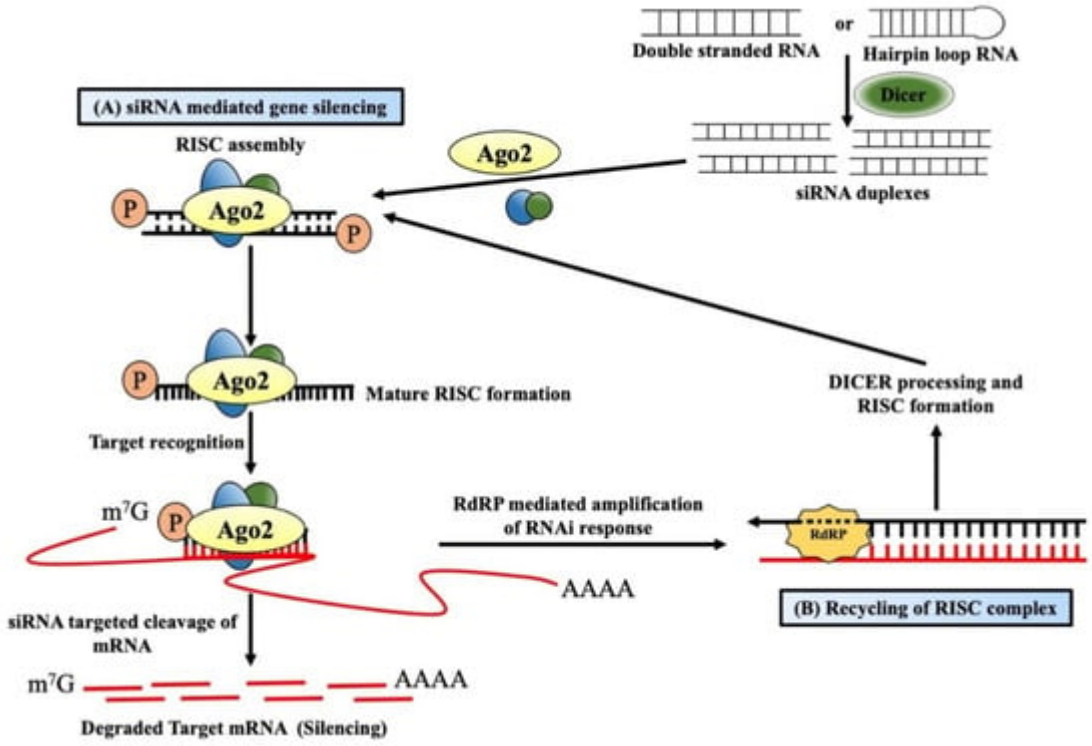

Figure 1. Mechanism of short-interfering RNA-mediated gene silencing. (**A**) Long double-stranded RNAs/hairpin loop RNAs from alien genes are processed into short interfering RNAs (siRNAs) by the Dicer/TRBP (the human immunodeficiency virus transactivating response RNA binding domain) complex and finally become incorporated into RNA-induced silencing complex (RISC). One strand (passenger strand) is degraded from the RNA duplex, and the other strand (guide strand), along with argonaute 2, forms the active RISC. The guide strand guides the active RISC to target and cleaves the complementary mRNAs into the cytosol, resulting in gene silencing. (**B**) RNAi response in plants and worms generally becomes amplified by the RNA-dependent RNA polymerase enzymes (RdRPs). RdRPs and RISC use targeted mRNAs as a template to generate double-stranded RNAs dsRNAs, which then are processed by the Dicer into secondary siRNAs. These siRNAs eventually amplify the RNAi effect in the system.

2. RNA-Induced Silencing Complex: The Versatile Gene Silencing Complex

Although there are diverse ways to regulate gene expressions using RISC, two significant incidents are common for all types. Firstly, every RISC should comprise one argonaute protein family member, and secondly, at its core, a small RNA should guide the RISC to target mRNAs through Watson–Crick base pairing [19]. Each class of small RNAs in eukaryotes, such as siRNA/miRNA/piRNA (only in animals), together with the AGO protein family, forms the ribonucleoprotein RISC. Our current understanding of RISC (from birth to death) has been summarized here.

The Argonaute family of proteins is at the heart of RISC-mediated gene regulation [20][21]. There are four functional domains in AGO proteins: PIWI-AGO-Zwille (PAZ), Middle (MID), N-terminal (N), and PIWI (**Figure 2**) [20][22]. There are two linkers (L1, L2), out of which L1 connects the N and PAZ domains, whereas L2 supports the N-L1-PAZ

structure connecting it to the MID-PIWI lobe [23][24]. The PAZ domain of AGO proteins contains a pocket that interacts with and binds the guide strand from the 3′ end [25]. A conserved sequence of a catalytic tetrad (Asp-Glu-Asp-His/Asp) can be found in the PIWI domain of some AGO proteins that are responsible for the target mRNA degradation. This catalytic domain is also responsible for the cleavage of some passenger strands before it gets ejected [22]. In the initial phase of RISC assembly, a small RNA duplex is loaded onto an empty AGO protein with the help of Hsp70/Hsp90 chaperons and forms the pre-RISC [26]. One of the strands, which is less stable and likely to be adenine/uridine (AU) rich, is preferably able to be the guide strand. This type of strand selection is asymmetric and generally depends on the difference in the thermodynamic stability of two ends of the RNA duplex [27][28]. At the initial stage of passenger strand separation, the N domain disrupts the 3′ end base-pairing of the guide strand to help open the RNA duplex [29]. Then, the passenger strand is sliced at a position opposite the guide strand's 10th and 11th nucleotide (g10 and g11) by the catalytic activity of the AGO protein [10][11][30]. After passenger strand separation, the mature RISC binds to the targeted mRNA guided by the guide strand and either slices it directly or induces translational repression by recruiting necessary proteins [31][32]. Although AGO and small RNAs are short-lived, once the RISC is formed, those two tend to be long-lived [33][34]. Generally blank/unloaded AGO proteins are degraded by the autophagy pathway [35][36], whereas the target RNA-directed miRNA degradation (TDMD) targeted RISC are degraded by the ubiquitin–protease system [37][38].

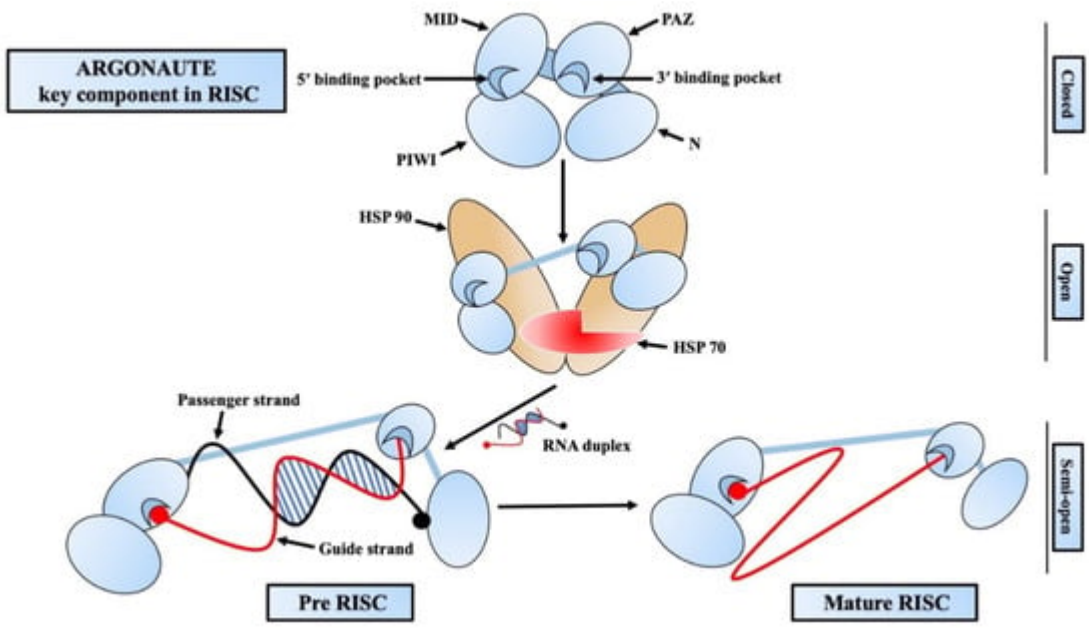

Figure 2. Mechanism of RNA-induced silencing complex (RISC) assembly. Argonaute has four functional domains: PIWI-AGO-Zwille (PAZ), Middle (MID), N-terminal (N), and PIWI (Piwi/Argonaute/Zwille). An empty argonaute loads an RNA duplex with the help of HSP70/HSP90 and forms the pre-RISC. The passenger strand (black strand) is ejected from the pre-RISC, and the guide strand (red strand) and argonaute form the mature/active RISC that start targeting the complimentary mRNAs to assert gene silencing.

3. Short Interfering RNA Mediated Silencing in Plants

The siRNA-mediated gene silencing mechanism has been exploited extensively over the years for crop protection against biotic stresses and as a platform for overall crop improvement. RNAi-mediated silencing requires the design of a hairpin loop structure containing both the sense and anti-sense strand of the targeted gene separated by an intron sequence. Such a construct will create hpRNAs in plants that are cleaved by Dicer and form siRNAs, which trigger the RNAi pathway to silence the targeted gene [39]. Pests such as insects, nematodes, viruses, and bacteria pose a severe threat to agricultural produce. RNAi has been used to make pest-resistant crops by targeting essential genes in the pests and silencing them by host plant-mediated RNAi (host-induced gene silencing—HIGS) [40].

The two-spotted spider mite is one of the deadliest plant pests; it can attack more than three thousand crops and feeds mainly on Chinese cabbage. RNAi-mediated targeting against the *COPB2* gene of this pest resulted in an almost 100% mortality rate [41]. Similarly, transgenic potato plants expressing the molting-associated *EcR* gene showed enhanced resistance against the deadly Colorado potato beetle [42]. The root-knot nematode *Meloidogyne incognita* causes colossal damage to agricultural products worldwide. RNAi-mediated simultaneous silencing of *Mi-flp1*, *Mi-flp12*, and *Mi-flp18* genes resulted in enhanced resistance against this nematode [43]. An extensive list of such siRNA-mediated gene silencing to improve desirable traits in plants is listed. Because RNAi-derived plants have been categorized as genetically modified crops (GM crops) and have been the subject of bitter controversy, the exogenous application of RNAi-inducing dsRNA-based bio-pesticides is gaining popularity, as it provides a non-transgenic approach [44]. Tenllado et al. first reported the effective foliar application of dsRNAs targeting the Alfalfa mosaic virus (AMV), Tobacco etch virus (TEV), and Pepper mild mottle virus (PMMoV) in 2001 [45]. However, the authors did mention that the commercial success of such topical application of dsRNAs will depend on two critical parameters: cost-effectiveness and an optimized mode of delivery. In recent years, several studies have been conducted to deliver such topical solutions of dsRNAs with impeccable efficiency and target specificity [45][46][47]. Topically applied dsRNA-based biopesticides have high species specificity, low levels of toxicity, and a minimal environmental effect compared to traditional pesticides. If their distribution and usage can be regulated in a precautionary way, dsRNA-based biopesticides can revolutionize the integrated pest-management system [48].

4. Tweaking the siRNAs to Improve Desirable Traits in Plants

siRNAs are well known for their silencing role in the case of viral RNAs. They play a master role in regulating plant defense machinery against potential pathogens such as bacteria, viruses, fungi, oomycetes, and other parasitic plants. Recent research has provided evidence of the ability of siRNAs to suppress fungus and oomycetes by silencing specific pathogen genes related to pathogenesis. Thus, scientists have precisely concluded that siRNAs are a potential concoction of diverse gene sequences and are used as a “shotgun” which targets random genes of the pathogen with great efficiency [49]. After discovering the antiviral factor, virus-derived siRNAs (vsiRNAs) in tobacco infected with potato virus, there has been a breakthrough in plant immunity. These viral dsRNAs are directly targeted by plant DCLs, resulting in 21–24 nt long primary vsiRNAs. It has been established that these 21 nt long vsiRNAs specifically silence the detrimental viral RNAs through Post Transcriptional Gene Silencing (PTGS) [50]. The functions of vsiRNAs can also be categorized into two parts. One part is when the vsiRNAs

degrade the intruder viral genome and render antiviral tolerance to plants. On the other side, some vsiRNAs silence host gene expression and manipulate host resistance towards viral attack. During tomato yellow leaf curl virus (TYLCV) infection, it has been observed that vsiRNAs are utilized by TYLCV to silence *SILNR1*, a long noncoding RNA (lncRNA) associated with antiviral defense. vsiRNAs obtained from wheat yellow mosaic virus (WYMV) down-regulate host genes and activated broad-spectrum host immunity [51]. Another category of siRNA, which was first seen in Arabidopsis, which also participates in antiviral defense, is virus-activated siRNA (vasiRNA) [52]. In the case of antiviral defense, the vsiRNAs are generated from the viral genome, which safeguards the plants by destroying the viral RNA. However, in the case of non-viral plant pathogens, endogenous siRNA-orchestrated gene silencing is instantly triggered to alter the gene expression associated with plant immunity [49]. The components of the siRNA pathway interact among themselves and others to orchestrate plant immunity. RDR6 is essential for plant immunity because it aids in producing secondary siRNAs and silencing signals. For example, in rice, *shl2-rol*, which is a mutant line of the rice gene *OsRDR6* results in severe infection symptoms when the plant is attacked by *Xanthomonas oryzae* PV. *oryzae*, thus proving the importance of RDR6-dependent siRNAs in rendering tolerance against bacteria [53].

Artificial siRNAs are produced in plants via Host-Induced Gene Silencing (HIGS) to silence the deadly pathogen genes causing infection. A well-tested example is transgenic barley and wheat, which express artificial siRNAs that target gene *Avra10* and display increased resistance to *Blumeria graminis*, the causative agent of powdery mildew disease [54]. HIGS via engineered dsRNA resists parasitic plants. This strategy has been demonstrated in transgenic tobacco that expresses dsRNA against transcription factors controlling haustoria development and reduced vigor in *Cuscuta patagonia*. Similarly, *Orobanche aegyptiaca* has been recorded to grow and feed on tomatoes. The above examples highlight host-produced synthetic siRNAs' significant role in controlling parasitic plants.

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