Chapter

RNA Interference: An Overview

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Abstract

In the course of transgenic experiments on the nematode *Caenorhabditis elegans*, RNA interference, usually abbreviated as RNAi, was discovered first. It is a gene-silencing effect and is found to be widely distributed in eukaryotes. It was observed that control injections of sense RNA were just as effective as antisense RNA, directed at specifically inhibiting target genes in *C. elegans* by the injection of antisense RNA during an experiment causing the reduction or elimination of expression from the gene under investigation. Subsequently, by injecting double-stranded RNA (dsRNA) corresponding to the target gene, it was discovered that the effect could be most potently elicited, and contamination of the single-stranded RNA (ssRNA; either sense or antisense) by traces of dsRNA could explain the earlier results. By post-transcriptional mechanism, substantial or complete inhibition of expression from any gene can be done using dsRNA corresponding to part or all of the mature mRNA from any given gene. An attempt was made here to describe the basic underlying molecular mechanism of RNAi, the methodology and various experimental requirements, and its advantages and disadvantages. In relation to CRISPR/Cas9 technology, the future prospects of virus-induced gene silencing (VIGS) are considered finally. For the cutting-edge CRISPR/Cas9 genome editing technology, VIGS has emerged as the preferred delivery system besides using it to overexpress or silence genes.

Keywords: RNAi, virus-induced gene silencing (VIGS), plant virus, CRISPR/Cas system

1. Introduction

A biological process where the expression of a particular gene is inhibited when specific mRNA molecules targeted and destructed by RNA molecule is known as RNA interference (RNAi). RNAi is otherwise called posttranscriptional gene silencing (PTGS), co-suppression, and quelling. The RNAi's disclosure was absolutely good fortune. The concept of RNAi for the first time came into the existence while the study of transcriptional inhibition by antisense RNA expressed in transgenic *Petunia* plant [1]. Scientists were trying to introduce these plant additional copies of chalcone synthase gene responsible for darker pigmentation of flowers. White or less pigmented flowers were observed instead of darker flowers, indicating the suppressed/decreased expression of endogenous chalcone synthase gene [1, 2] when intended to make more corresponding gene products. This suggests a downregulation of

endogenous gene by the event posttranscriptional inhibition due to their mRNA degradation [3, 4]. Just after the discovery of plant defense mechanism against virus, where it was believed that plant encodes short, noncoding region of viral RNA sequences, silencing of target genes by RNA interference technology came into the lime light, which after contamination perceives and debases viral mRNA. These short and noncoding RNA arrangements may be against viral DNA/RNA polymerase and other significant genes essential for viral contamination and multiplication. On the topic of the above idea, plant virologist brought short nucleotides sequence into the viruses, and expression of target genes in the infected plants was seen as suppressed [5, 6]. This most mainstream marvel is known as "virus-induced gene silencing" (VIGS) and gets a blast of the time of biotechnologists. Craig Mello and Andrew Fire, after a year later in 1998, worked in the laboratory to study the effect of RNAi in *Caenorhabditis*. The term RNAi was coined by these two scientists for the first time, and they were awarded the Nobel Prize in 2006 [7]. After this incredible disclosure of double-stranded RNA (dsRNA) as an amazingly strong trigger for gene silencing, it turned out to be sensible to unwind the component of RNAi activity in different biological systems [8, 9]. Protein apparatus important for gene silencing was found in *C. elegans* without precedent for 1999, and thorough examination shows that normal principal process must be worked all through the eukaryotes, for example, fungi, Drosophila, and plants [10]. Small RNA extending long from 21 to 23 nucleotides created from dsRNA in cell separates and could behave as de novo silencing trigger for RNAi in cell extracts free of dsRNA treatments. They reasoned that short 21–23 nucleotide siRNA are the result of Dicer and RNA-induced silencing complex (RISC) [11–13].

The short RNA molecules, a key to RNA interference technology, are of two sorts: (I) microRNA (miRNA) and (II) small interfering RNA (siRNA). miRNAs are endogenous or intentionally expressed product (organism own genome product), though siRNAs are inferred result of exogenous cause, for example, virus and transposon. Both have distinctive forerunner, for instance, miRNA processed from stem-loop with partial complementary dsRNA though siRNA shows up from fully complementary dsRNA [14]. In spite of these differences, both short nucleotides are very much related in terms of their biogenesis and mode of action [15].

2. Basic components of RNAi

2.1 Dicer: a gateway into the RNA interference

Dicer, a member of RNase III family proteins with dsRNA-specific nuclease activity and it act as a primary candidate for biogenesis of siRNA during gene silencing. These enzymes have a few basic motifs spread all through the polypeptide affix from N-end to C-end, which is liable for their productive execution [15]. RNase III proteins are portrayed by the spaces all together from N- to C-end: a DEXD domain, a DUF283 domain, a Piwi/Argonaute/Zwille (PAZ) domain, two RNase III domains, and a dsRNA binding domain. Aside from ribonuclease explicit PAZ domain, Dicer do have helicase domain, and their capacity has been embroiled in preparing long dsRNA substrate [16]. Out of these five significant domains, PAZ and RNase III are basic for exact extraction of siRNA from dsRNA forerunner [17]. The duplex RNA ends with three nucleotides overhang, bringing about extending of two helical turns along the outside of the protein perceived by PAZ domain. This prompts the cleavage of each out of the two strands in turn by two diverse RNase III

domains independently. The last product after Dicer activity is 21–23-nt-long fragments with two nucleotides overhang at 3′ end, which currently go about as a substrate for RISC [14]. Current finding recommends that PAZ domain is fit for restricting the precisely two nucleotide 3′ overhang of dsRNA, while the RNase III catalytic domains structure a pseudo dimer around the dsRNA to start cleavage of the strands. This results in a functional shortening of the dsRNA strand. The separation between the PAZ and RNase III domains is controlled by the angle of the connector helix and impacts the length of the microRNA product [18]. In some of the organism, just one copy of Dicer is answerable for the processing of both miRNA and siRNA; however in Drosophila, Dicer 1 is exclusively dedicated for miRNA biogenesis, while Dicer 2 is utilized for siRNA track [14]. Other variants of Dicer are characterized by the absence of ATPase domain or PAZ domain or RNA binding domains. Although functional ATPase domain is not very necessary for the action of Dicer to the substrate molecules, studies also give a clue that ATPase domain is very critical for switching/movement of both RNase III domains, and biochemical studies indicate mutation in ATPase domain leads to the abolishment of siRNA procession [14].

2.2 RISC: at the center of RNA interference

RISC is a generic term for a family of heterogeneous molecular complexes that can be programmed to target almost any gene for silencing. In the cytoplasm of a eukaryotic cell, RISC programming is triggered by the appearance of dsRNA. RISC is a multiprotein complex composed of ribonucleoproteins (Argonaute protein), incorporating one strand of dsRNA fragments (siRNA, miRNA) to the target transcripts. Two proteins of ~100 kDa were also identified that corresponded to Argonaute 1 and Argonaute 2 (Ago1 and Ago2). A variety of different ribonucleoproteins, ranging from modest size (150 kDa) up to 3 MDa particle termed "holo-RISC," have been revealed by the biochemical isolations of RISC, and many other intermediate sizes have also been observed [19–21]. A large number of RISC-associated proteins have been reported from recent research which mainly includes Argonaute proteins and RISC-loading complex. Both these components assembled together to perform its functions efficiently. RISC-loading complex is basically made up of Dicer, Argonaute, and TRBP (protein with three double-stranded RNA binding domains) [22] that identified a 500 kDa insignificant RISC by portraying proteins that copurified with human Dicer. Two proteins were seen as related with Dicer, Ago2, and TRBP (the HIV trans-activating response RNA-binding protein) [22]. Paralelly, the minimal RISC, sufficient for target RNA recognition and cleavage efficiently, was demonstrated to be simply an Argonaute protein bound to a small RNA [23]. Argonaute proteins are universally found in plant, animal, many fungi, protista, and even in some archaea also. Albeit every AGO protein harbor PAZ, MID (middle), and PIWI domains, they are isolated into three groups based on both their phylogenetic connections and their ability to tie to small RNAs. Group 1 individuals are alluded to as AGO proteins which bind to miRNAs and siRNAs. Group 2 members are referred to as PIWI proteins which bind to PIWI-interacting RNAs (piRNAs). Group 3 individuals have been depicted uniquely in worms, where they tie to secondary siRNAs. AGOs are large proteins (ca 90–100 kDa) comprising one variable N-terminal area and rationed C-terminal PAZ, MID, and PIWI domains. Experiments with bacterial and animal AGO proteins have clarified the roles of these three domains in small RNA pathways. The MID domain ties to the 5′ phosphate of small RNAs, while the PAZ domain perceives the 3′ end of small RNAs. The PIWI domain adopts a collapsed structure

like that of RNaseH proteins and shows endonuclease action, which is done by a functioning site typically conveying an Asp-Asp-His (DDH) motif [24].

The presence of these proteins has also been reported in prokaryotes, but their function in lower organisms is still a mystery. Among eukaryotes, number of Argonaute gene ranging from a single copy to dozens of copies (even more than two dozens) is found to be observed. Multiple copies (paralogous proteins) of Argonaute proteins in *C. elegans* reflect their functional redundancy, and their evolutionary significance remains unknown. Studies suggest genes for Argonaute proteins are ample to recompense for one another [25]. In association with siRNA, the Argonaute binds to the 3′-untranslated area of mRNA which prevents the creation of proteins in a few different ways. The enrollment of Argonaute proteins to focused mRNA can induce mRNA degradation. The Argonaute-miRNA complex can likewise impact the development of functional ribosomes at the 5′ end of the mRNA. The complex contends with the translation initiation factors and/or potentially repeals ribosome to get together. By recruiting cellular factors such as *peptides* or posttranslational modifying enzymes, the Argonaute-miRNA complex can adjust protein production, degrading the growth of polypeptides [26].

The Argonaute superfamily can be separately partitioned into three subgroups: the Piwi clade that ties to piRNAs, the Ago clade that associates with miRNAs and siRNAs, and a third clade that has just been found and portrayed in nematodes so far [27]. All gene regulatory phenomena including ~20–30 nt RNAs are thought to require at least one Argonaute protein, and these proteins are the central, characterizing segments of the different types of RISC. The double-stranded products of Dicer enters into a RISC assembly pathway that involves duplex unwinding, culminating in the stable association of only one of the two strands with the Ago effector protein [14, 15]. Thus, through Watson-Crick base pairing, one guide strand directs target recognition, while the other strand of the first little RNA duplex, known as the passenger strand, is disposed of. There are eight AGO relatives in human, some of which are examined seriously. Despite the fact that AGO1–AGO4 are equipped for stacking miRNA, endonuclease action and, however, RNAi subordinate gene silencing are solely found with AGO2. The uniqueness of AGO2 is presumed to arise from either the N-terminus or the spacing region linking PAZ and PIWI motifs, considering the sequence conservation of PAZ and PIWI domains across the family. In plants, a few AGO families additionally draw a tremendous effort of study. AGO1 is unmistakably engaged with miRNA-related RNA degradation and assumes a central role in morphogenesis. In certain organisms, it is carefully required for epigenetic silencing. Strangely, it is managed by miRNA itself. AGO4 does not include in RNAi-coordinated RNA degradation, yet it includes in DNA methylation and other epigenetic regulation through small RNA (siRNA) pathway. AGO10 is associated with plant development. AGO7 has a distinct function from AGO1 to AGO10 and is not included in gene silencing actuated by transgenes. Rather, it is identified with developmental timing in plants [15, 28]. At the cell level, Ago proteins diffusely restrict in the cytoplasm and nucleus and sometimes, likewise at particular, foci which incorporate processing bodies (P-bodies) and stress granules. The subsequent clade, Piwi (named after the Drosophila protein PIWI, for P-component instigated weak testis), is communicated in germline cells most bounteously and has the capacity in the silencing of germline transposons. The means by which members acquire guide RNAs (gRNAs) is a major biochemical difference between Argonaute clades. In the cytoplasm, Ago guide RNAs are produced from dsRNA by a particular nuclease named Dicer. Individuals from the Piwi clade are thought to frame direct RNAs in a "ping-pong" component in which the objective RNA of one Piwi protein is severed and turns into the guide RNA of another Piwi protein. Maternally acquired guide piRNAs are accepted to start this gene-silencing cascade.

Class 3 Argonautes get direct RNAs by Dicer-interceded cleavage of exogenous and endogenous long dsRNAs [27, 29, 30].

The hall mark domains of Argonaute proteins are N-terminal PAZ (like Dicer enzymes and offer basic developmental cause), mid domain and C-terminal PIWI domain, an interesting to the Argonaute superfamily proteins. The PAZ domain is named after the revelation of proteins PIWI, AGO, and Zwille, whereby it is found to be conserved. The PAZ domain interacts with 3′ end of both siRNA and miRNA in a sequence-independent manner, and finally it hybridizes via base-pairing interaction with the target mRNA, leading to the cleavage or translation and inhibition [31]. PIWI domain has structural resemblance with RNaseH which is very essential for RNA backbone cleavage. The active site which coordinates with divalent metal ion and provides binding energy for catalysis is composed of triad amino acids, aspartate–aspartate–glutamate. PIWI domain participates in interaction with the Dicer via one of the RNase III domains in few Argonaute proteins [15]. A MC motif is available between the Mid and PIWI domain, which is believed to be engaged with collaboration sites for the 5′ cap of siRNA/miRNA and control their translation [26]. The general structure of Argonaute is bilobed, with one flap comprising the PAZ domain and the other projection comprising the PIWI domain which is flanked by N-terminal (N) and center (Mid) domains. The Argonaute PAZ domain has RNA 3′ end binding activity and is used in guide strand binding revealed by the co-crystal structures. The other end of the guide strand engages a 5′ phosphate binding pocket in the mid domain, and the remainder of the guide tracks along a positively charged surface to which each of the domains contributes. As expected for a protein that can accommodate a wide range of guide sequences, the protein-DNA contacts are dominated by sugar-phosphate backbone interactions. Guide strand that consists of 2–6 nucleotides which are important especially for target recognition is stacked with their exposed Watson-Crick faces and available for base pairing [32].

3. Working principle of RNAi

The RNAi pathway, ubiquitous to most of the eukaryotes consists of a short RNA molecule that binds to specific target mRNA to form a dsRNA hybrid and inactivates the mRNA by preventing it from producing a protein. It also influences the development of organisms, apart from their role in defense against viruses and protozoans. During RNAi, the dsRNA, introduced into cells by viral infection or artificial expression or formed in cells by DNA- or RNA-dependent synthesis of complementary strands, is processed to 20 bp double-stranded siRNAs containing 2-nt 3′ overhangs [33]. The siRNAs are then incorporated into an RNA-induced silencing complex, recognize the sequences fully complementary to the siRNA, and mediate the degradation of mRNAs (**Figure 1**) [34].

3.1 Initiation: processing of precursor dsRNA

In the cytoplasm, RNAi pathway, an RNA-dependent pathway, can be initiated by either exogenous or endogenous short dsRNA particles. The forerunner of siRNA, named as essential siRNA or pri-siRNA, creases back to frame a long stem circle structure (endogenous source dsRNA), at the cleavage site, leaving two 3′ overhang nucleotide and 5′ phosphate group [35]. Inside the nucleus, Drosha and Pasha, in case of miRNA, are responsible for trimming the end of stem-loop like pri-miRNA, leading to the generation of pre-miRNA. Now, this pre-miRNA is transported to the cytoplasm with the help of Ran-GTP mediated exportin-5 nuclear transporter, where Dicer chops the dsRNA into mature miRNA [36].

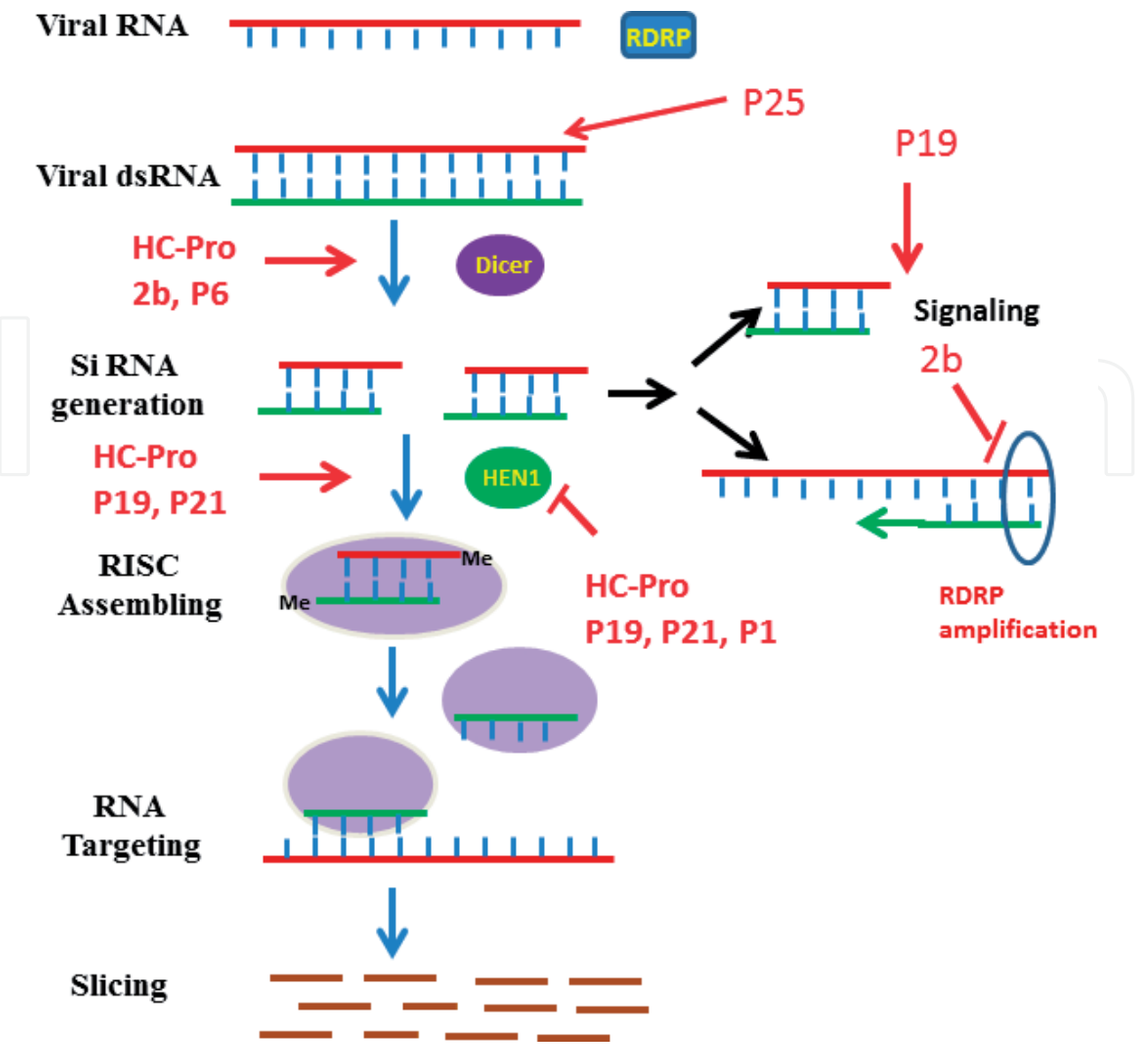

Figure 1.
Viral RNA silencing in plant and its counter defense.

Processing of exogenous RNAs is cytoplasmic that leads to the biogenesis of siRNA only requires Dicer but not Drosha. Dicer contains two RNase III domains, one helicase domain, one dsRNA binding domain, and one Piwi/Argonaute/Zwille domain. The PAZ domain known to be very essential for RNAi is also found in Argonaute family proteins. The present finding proposes that the binding of Dicer as far as possible of dsRNA is unmistakably more impressive than inner binding. Dicer will connect with a current end of dsRNA and removes ~21 nucleotides from the end, shaping another end with two 3′ overhangs. A pool of 21-nt-long small RNA with two 3′ overhangs nucleotides will be generated from long dsRNAs, as a result of this stepwise cutting [37]. A few organisms contain more than one Dicer genes, with every Dicer specially processing dsRNAs from various sources. *Arabidopsis thaliana* has four Dicer-like proteins, out of which DCL-1 participates in microRNA development, DCL-2 especially processes dsRNA from plant virus and DCL-3 generates small RNAs from endogenous repeated sequences. Interestingly, only one Dicer gene is encoded by most of the mammals [38].

3.2 Selection of siRNA strand and assembly of RISC

The products of dsRNA and pre-siRNA processing by Dicer are 20 bp duplexes with 3′ overhangs. However, functional RISCs that consist of miRNAs and siRNAs must be single stranded for matching with the target RNA. How are the duplexes changed over to single-chain structures and how is a right (e.g., antisense or

"direct") strand chosen for stacking onto the RISC? The later inquiry is of reasonable significance in light of the fact that in order to knock down genes, artificial siRNAs can be directly used to trigger RNAi. Sequence analysis of the duplexes formed by pre-siRNA processing by Dicer and measurements of the potency of different double- and single-stranded siRNAs have demonstrated that the strand fused into the RISC is commonly the one whose 5′ end is the thermodynamically less steady end of the duplex [39]. Recent studies suggest that, in Drosophila, the Dcr-2–R2D2 heterodimer senses the differential stability of the duplex ends and decides which siRNA strand should get selected. Dicer binds to a less stable and R2D2 to a more stable siRNA end that is demonstrated by photocross-linking to siRNAs containing 5-iodouracils at different positions. Argonaute proteins are the most conserved members of RISC, which are essential most for RISC functions. Argonaute proteins are highly rich in basic amino acids, and in plants, these residues are basically responsible for cross-linking with the guide RNA [40]. Argonaute proteins are characterized by the presence of two homology regions, the PAZ domain and the PIWI domain (RNase H like functional motif). PAZ domain specifically recognizes the unique structure of two 3′ nucleotides overhangs of siRNAs and also appears in Dicer proteins. In Argonaute proteins, PIWI domain recognizes 5′ phosphate group and therefore is required for siRNA to assembly into RISC. Endogenous kinase rapidly phosphorylates siRNA, lacking phosphate group in 5′ end [41]. Transfer of Dicer-processed dsRNA to RISC is mediated by several unknown proteins. RISC needs an ATP-dependent process for activation, which helps in loosening up siRNA duplex, leaving just single-stranded RNA joining the dynamic type of RISC. Near studies on solidness among functional and nonfunctional siRNA demonstrate that the 5′ antisense regions of the practical siRNAs were less thermodynamically stable than the 5′ sense districts, giving a premise to their specific passage into the RISC. Through Watson-Crick base paring, the strand remained within the RISC function as a guide to locate target mRNA sequence, while during the loading process, the other strand of duplex siRNA is either cleaved or discarded. The only member of the Argonaute subfamily of proteins, the endonuclease Argonaute 2 with observed catalytic activity in mammalian cells, is liable for this cutting action. Severed transcripts will experience resulting degradation by cell exonucleases. During this procedure, the guiding strand of siRNA duplex inside RISC will be unblemished and thusly catalytically permit RISC function. This strong cleavage pathway makes it an extremely appealing technique for decision for potential restorative utilizations of RNAi [42]. It is still a matter of debate, whether siRNA-mediated regulation has an impact on initiation, elongation, or termination or whether it acts co-translationally. For instance, human Ago2 ties to m7GTP and in this way can contend with eukaryotic translation initiation factor 4E (eIF4E) for binding to them the 7GTP-top structure of mRNA; the relationship of human Ago2 with eIF6 and large ribosomal subunits additionally recommends an early advance of translation repressed by miRNAs. In any case, miRNAs and AGOs are found to be related with polysomes, proposing that at least in some cases, inhibition occurs after initiation [24].

The majority of the miRNAs hybridize to target mRNA with a near-perfect complementarity in plants and through a similar, if not identical, mechanism used by the siRNA pathway mediate an endonucleolytic cleavage. While in animal, miRNA interacts only with 3'UTR of mRNA (For ex; lin-4) and regulated expression of proteins negatively. The focal bungle between miRNA and mRNA hybridization is accepted to be answerable for the absence of RNAi-interceded mRNA cleavage occasions (e.g., absence of RISC-intervened mRNA debasement). At long last, miRNA-mRNA complex related with Ago proteins moves to processing body, where mRNA at long last is debased by RISC-free pathway [43, 44]. RNAi that interceded the

silencing of genes is not constrained to the posttranscriptional level as it was. SiRNA can also trigger de novo DNA methylation and transcriptional silencing; it has been shown in plants. Recent evidence suggests that in the genomes of certain species, siRNAs can inactivate transcription through direct DNA methylation and other types of covalent modification. Several studies also demonstrated that for the formation and maintenance of higher-order chromatin structure and function, RNAi machinery present in the fission yeast *S. pombe* plays a critical role. It is hypothesized that expression of centromeric repeats results in the formation of a dsRNA that is cleaved by Dicer into siRNAs that direct DNA methylation of heterochromatic sites and regulate the expression of genes [45, 46]. Suppressors of posttranscriptional RNA silencing are encoded by many plant and some animal viruses that interfere with the accumulation or function of siRNAs. Recent crystallographic studies have revealed how the p19 suppressor protein of *Tombusviridae* elegantly and effectively sequesters siRNAs aimed at destroying viral RNA [47, 48].

In plant defense against pathogen invasion, RNA silencing functions as a natural immunity mechanism [49], and many viruses have evolved to express virus silencing repressor (VSR) proteins to counteract host antiviral RNA silencing. At molecular level, some of the virus-silencing repressors were studied, for example, 2b of cucumber mosaic, P69 of the turnip yellow mosaic virus (TYMV), and HC-Pro of the turnip mosaic virus (TuMV), in Arabidopsis. Without a doubt, P19 protein of tombusviruses, the most popular VSR up until this point, forestalls RNA silencing by siRNA sequestration through binding ds siRNA with a high affinity [50]. Crystallographic examines have uncovered that P19 structures are a tail-to-tail homodimer, which acts like a subatomic caliper, estimating the length of siRNA duplexes and restricting them in a sequence autonomous way, choosing for the 19-bp-long dsRNA region of the common siRNA [48]. It is also confirmed through latest findings that the spread of the ds siRNA duplex hindered by P19 is recognized as the sign of RNA silencing [51].

Different VSRs, for example, the tomato aspermy cucumovirus 2b protein or B2 of the insect-infecting Flock House infection, likewise tie ds siRNA in a size-explicit way; all things considered auxiliary examinations have demonstrated that their methods of binding siRNAs do not impart any closeness to P19 [52].

In agroinfiltration assays, two viral proteins that were recognized appeared to restrain the processing of dsRNA to siRNAs: P14 of Pothos latent aureusvirus and P38 of turnip crinkle virus (TCV). As of late, it was found that the activity of the P38 protein happens through AGO1 binding and that it meddles with the AGO1-dependent homeostatic network, which prompts the hindrance of Arabidopsis DCLs [53]. The P6 VSR of the cauliflower mosaic virus (CaMV) studies has shown to interfere with vsiRNA processing, in addition to P14 and P38. Previously, P6 was portrayed as a viral translational trans-activator protein basic for virus biology. Critically, P6 has two importin-alpha-dependent nuclear localization signals, which are obligatory for CaMV infectivity. An ongoing disclosure demonstrated that one of the nuclear functions of P6 is to stifle RNA silencing by interacting with dsRNA-restricting protein 4, which is required for the functioning of DCL-4.

4. Virus-induced gene silencing

Van Kammen termed "virus-induced gene silencing" first of all to describe the phenomenon of recovery from virus infection [54]. Though, the term has since been applied almost exclusively to the technique involving recombinant viruses to knock down the expression of endogenous genes [55, 56]. Around the world, RNA silencing has become a major focus of molecular biology and biomedical research.

Plant biologists have adopted numerous methods to engineer resistant plants that reduce the losses caused by plant pathogens. During the last two decades, RNA silencing-based resistance has been a powerful tool that has been used to engineer resistant crops, among them. In view of this system, various methodologies were created. Virus-induced gene silencing is a virus vector technology that uses an RNA-intervened antiviral defense mechanism. In plants, infected with unmodified viruses, the system is explicitly focused against the viral genome. However, with virus vectors carrying inserts derived from host genes the process can be additionally targeted against the corresponding mRNAs. VIGS has been generally utilized in plants for investigation of gene function and has been adjusted for high-throughput functional genomics. Most uses of VIGS have been studied in *Nicotiana benthamiana*, up to this point. In any case, in other plants including *Arabidopsis*, new vector systems and methods are being developed that could be used. VIGS also helps in the identification of genes required for disease resistance in plants. When VIGS is used in the analysis of other aspects of plant biology, these methods and the underlying general principles are also applied.

When a plant virus infects a host cell, it activates an RNA-based defense that is targeted against the viral genome. In the virus-infected cells, dsRNA is thought to be the replication intermediate that causes the siRNA/RNase complex to target the viral single-stranded RNA. The viral ssRNA would not be a target of the siRNA/RNase complex in the initially infected cell because this replication intermediate would not have accumulated to a high level. However, the viral dsRNA and siRNA would become more abundant, as the rate of viral RNA replication increases in the later stages of the infection. Eventually, the viral ssRNA would be targeted intensively, and virus accumulation would slow down [57]. Many plant viruses encode proteins that are suppressors of this RNA silencing process. These suppressor proteins would not cause complete suppression of the RNA-based defense mechanism as they would not be produced until the virus had started to replicate in the infected cell. Nonetheless, these proteins would impact the final steady-state level of virus accumulation. Strong suppressors would permit virus aggregation to be drawn out and at a significant level. Alternately, if a virus gathers at a low level, it could be because of the weak suppressor activity [58]. The dsRNA replication intermediate would be prepared with the goal that the siRNA in the infected cell would compare to parts of the viral vector genome, including any nonviral insert. Thus, the siRNAs would target the RNase complex to the corresponding host mRNA, if the insert is from a host gene and the symptoms in the infected plant would reflect the loss of the function in the encoded protein.

There are a few models that strongly support this way to deal with suppression of gene expression. In this manner, when tobacco mosaic virus (TMV) or potato virus X (PVX) vectors were adjusted to convey inserts from the plant phytoene desaturase gene, on the infected plant the photobleaching indications reflects the non-attendance of photoprotective carotenoid pigments that require phytoene desaturase. Thus, when the virus conveys additions of a chlorophyll biosynthetic enzyme, there were chlorotic side effects, and, with a cellulose synthase insert, the infected plant had modified cell walls [59]. Genes other than those encoding metabolic catalysts can likewise be focused by VIGS. For instance, if the viral supplement related to genes is required for virus opposition, the plant showed upgraded pathogen weakness. In one such model, the supplement in a tobacco rattle virus (TRV) vector was from a gene (EDS1) that is required for N-intervened protection from TMV. The virus vector-tainted N-genotype plant showed mediated TMV obstruction. The manifestations of a TRV vector conveying a verdant supplement show how VIGS can be utilized to target gene that directs advancement. *Leafy* is a gene required for bloom advancement. Loss-of-function *Leafy* mutants produce changed

blossoms that are phenocopied in the TRV-leafy-infected plants. Correspondingly, the effects of tomato golden mosaic infection vectors carrying parts of the gene for a cofactor of DNA polymerase shows, how VIGS can be utilized to target essential genes. The plants infected with this geminivirus vector were suppressed for division development in and around meristematic zones of the shoot [60].

RNAi via siRNAs has generated a great deal of interest in both basic and applied biology to exploit the ability to knock down any gene of interest. There are an expanding number of large-scale RNAi screens that are intended to recognize the significant genes in different biological pathways. As the ailment forms additionally rely upon the consolidated activity of different genes, it is normal that killing the action of a gene with explicit siRNA could deliver a restorative advantage to humanity. Based on the siRNA-mediated RNA silencing (RNAi) mechanism, several transgenic plants has been designed to trigger RNA silencing by targeting pathogen genomes. Based on the difference in precursor RNA, diverse targeting approaches have been developed for siRNA creation, including sense/antisense RNA, small/long hairpin RNA, and man-made miRNA antecedents. Numerous transgenic plants have been planned by virologists, expressing viral coat protein (CP), movement protein (MP), and replication-related proteins, demonstrating to be safe against contamination by the homologous virus. This sort of pathogen-determined resistance (PDR) has been accounted for in different infections including tobamovirus, potexvirus, cucumovirus, tobravirus, Carlavirus, potyvirus, and alfalfa mosaic virus bunches just as the luteovirus gathering [49, 61]. Transgene RNA silencing-intervened resistance is a procedure that is exceptionally connected with the amassing of viral transgene-inferred siRNAs. One of the disadvantages of the sense/antisense transgene approach is that the opposition is shaky and the component regularly brings about deferred obstruction or low adequacy/resistance. This might be because of the low collections of transgene-inferred siRNA in PTGS because of the defense system encoded by plants. Additionally, various infections, including potyviruses, cucumoviruses, and tobamoviruses, can check these systems by hindering this kind of PTGS. Thusly, the rich expression of the dsRNA to trigger productive RNA silencing gets significant for viable obstruction. To accomplish opposition, inverse repeat sequences from viral genomes were broadly used to frame hairpin dsRNA in vivo, including small hairpin RNA (shRNA), self-complementary hpRNA, and intron-spliced hpRNA. Among these techniques, self-complementary hairpin RNAs is isolated by an intron prone to evoke PTGS with the most elevated proficiency. The nearness of modified rehashes of dsRNA-induced PTGS (IR-PTGS) in plants likewise demonstrated high resistance against viruses. For the processing of primary siRNAs, IRP-TGS is not required for the formation of dsRNA; in any case, the plant RDRs are liable for the age of secondary siRNAs got from non-transgene viral genome, which further strengthens the adequacy of RNA silencing instigated by hpRNA, a procedure named RNA silencing transitivity. Among them, the most significant are sequence closeness between the transgene sequence and the difficult virus infection arrangement. Scientists have engineered several transgenic plants with multiple hpRNA constructs from different viral sources, or with a single hpRNA construct combining different viral sequence. At the same time, various viruses can be focused on, and the subsequent transgenic plants show a more extensive resistance with high viability. Notwithstanding the arrangement closeness, the length of the transgene sequence additionally adds to high resistance. As a rule, transgene sequence with a normal length of 100–800 nt gives viable obstruction [62, 63].

By mimicking the unblemished secondary structure or hairpin loop of endogenous miRNA antecedents, artificial miRNAs (amiRNAs) are planned and handled

in vivo to focus on the genes of intrigue. The technique of expressing amiRNAs was first adopted to knock down endogenous genes for functional analysis. The innovation is generally utilized in building antiviral plants and animals. Conventional with regular RNAi methodologies, amiRNAs have numerous preferences:

1. Owing to the short sequence of amiRNAs, a long viral cDNA fragment is not required; therefore, the full degree of off-target impacts are avoided, and the biosafety of transgenic crops is expanded, contrasted with siRNAs from long clip RNA.

2. Tissue- or cell-explicit take-out/downs of genes of intrigue can be acknowledged as a result of various tissue- or cell-explicit advertisers being utilized.

3. The casual interest on sequence length makes amiRNAs particularly valuable in focusing on a class of moderated genes with high succession likenesses, similar to a couple of exhibited genes, on the grounds that a short preserved grouping is all the more effectively found in these genes [64].

Viruses which have been altered and utilized for silencing the gene of intrigue are outlined in **Table 1** [65–86]. Tobacco mosaic virus is one of the changed viruses which were utilized for compelling pds gene silencing in *Nicotiana benthamiana* plants. TMV is the main changed virus for use of VIGS techniques to plant. Potential of VIGS for analysis of gene function was easily recognized when the viral delivery leads to downregulation of transcript of target gene through its homology-dependent degradation. Tobacco rattle virus was also modified to be a tool for gene silencing in plants. Using TRV vectors, VIGS has been effectively applied in *N. benthamiana* and in tomato. The critically preferred position of TRV-based VIGS in solanaceous species is the simplicity of presentation of the VIGS vector into plants. The VIGS vector is set between right border (RB) and left border (LB) locales of T-DNA and embedded into *Agrobacterium tumefaciens* [81, 82].

Another property of TRV is the more vivacious spreading everywhere throughout the whole plant including meristem, and disease manifestations of TRV are gentle. Strong duplicate 35S promoter and a ribozyme at C-terminus make modified TRV vectors such as pYL156 and pYL279 more efficient and spread faster. These vectors are also able to infect other plant species. In tomato, TRV-based vector has been used [43] for gene silencing. Recently, Pflieger et al. have indicated that a viral vector got from turnip yellow mosaic virus (TYMV) can prompt VIGS in *Arabidopsis thaliana*. VIGS of *N. benthamiana* utilizing potato virus X (PVX) was likewise accomplished. PVX-based vectors have more limited host range (only three families of plants are susceptible to PVX) than TMV based vectors (nine plant families show susceptibility for TMV) but PVX-based vectors are more stable compared to TMV. For VIGS studies, geminivirus-inferred vectors can be utilized particularly to examine the capacity of genes associated with meristem function. Tomato golden mosaic virus (TGMV) was utilized to silence a meristematic gene, proliferating cell nuclear antigen (PCNA) in *N. benthamiana*. The TGMV-based silencing vector had been utilized for likewise silencing of non-meristematic gene silencing. In plants, only with the help of other helper viruses for efficient gene silencing, satellite virus-based vectors are also used. This two-component system is called satellite virus-induced silencing system (SVISS) [60, 87]. Previously, barley stripe mosaic virus (BSMV) was developed for efficient silencing of *pds* gene, in barley. Then, this system was used for silencing of wheat genes. BSMV is a positive-sense RNA virus containing a tripartite (α, β, γ) genome. The modified γ of BSMV genome was used for plant

Virus/type	Group	Natural hosts	Silenced host species	Gene silenced	References
African cassava mosaic virus, DNA virus, bipartite	Begomovirus	*Manihot esculenta*	*N. benthamiana*, *M. esculenta*	*pds*, *su*, *cyp79d2*	[65]
Apple latent spherical virus RNA virus, bipartite	Cheravirus	Apple	*N. tabacum*, *N. occidentalis*, *N. benthamiana*, *N. glutinosa*, *Solanum lycopersicon*, *A. thaliana* Cucurbit species, several legume species	*pds*, *su*, *pcna*	[66]
Barley stripe mosaic virus RNA virus, tripartite	Hordeivirus	Barley, wheat, oat, maize, spinach	*Hordeum vulgare, Triticum aestivum*	*Pds*, *TaEra1*	[67, 68]
Bean pod mottle virus RNA virus, bipartite	Cucumovirus	*Phaseolus vulgaris*, *Glycine max*	*G. max*	*Pds*, *GmRPA3*	[69, 70]
Brome mosaic virus RNA virus, tripartite	Bromovirus	Barley	*Hordeum vulgare*, *Oryza sativa*, *and Zea mays*	*pds*, *actin 1*, *rubisco activase*	[71]
Cabbage leaf curl virus DNA virus, bipartite	Begomovirus	Cabbage, broccoli, cauliflower	*A. thaliana*	*gfp*, *CH42*, *pds*	[72]
Cucumber mosaic virus RNA virus, tripartite	Cucumovirus	Cucurbits, *S. lycopersicon*, *Spinacia oleracea*	*G. max*	*chs*, *sf30h1*	[73]
Pea early browning virus, RNA virus, Bipartite	Tobravirus	*Pisum sativum*, *Phaseolus vulgaris*	*P. sativum*	*pds*, *uni*, *kor*	[74]
Poplar mosaic virus RNA virus, monopartite	Carlavirus	Poplar	*N. benthamiana*	*gfp*	[75]
Potato virus X RNA virus, monopartite	Potexvirus	*Solanum tuberosum*, *Brassica campestris ssp. rapa*	*N. benthamiana*, *A. thaliana*	*gus*, *pds*, *DWARF*, *SSU*, *NFL*, *LFY*	[76]
Satellite tobacco mosaic virus RNA virus, satellite	RNA satellite virus	*Nicotiana glauca*	*N. tabacum*	*Several genes*	[77]

Virus/type	Group	Natural hosts	Silenced host species	Gene silenced	References
Tomato bushy stunt virus, RNA virus	Tombusvirus	*S. lycopersicon*, *N. benthamiana*	*N. benthamiana*	*gfp*	[78]
Tobacco curly shoot virus, DNA satellite-like virus	DNA satellite-like virus	*N. tabacum*	*N. tabacum*, *Solanum lycopersicon*, *Petunia hybrida*, *N. benthamiana*	*gfp*, *su*, *chs*, *pcna*	[79]
Tobacco mosaic virus RNA virus, monopartite	Tobamovirus	*N. tabacum*	*N. benthamiana*, *N. tabacum*	*pds*, *psy*	[80]
Tobacco rattle virus RNA virus, bipartite	Tobravirus	Wide host range	*N. benthamiana*, *A. thaliana*, *S. lycopersicon*	*pds*, *rbcS*, *FLO/LFY (NFL)* *Sllea4*	[81, 82, 83]
Tomato golden mosaic virus, DNA virus, bipartite	Begomovirus	*S. lycopersicon*	*N. benthamiana*	*su*, *luc*	[84]
Tomato yellow leaf curl China, DNA satellite	Begomovirus	*S. lycopersicon*	*N. benthamiana*, *S. lycopersicon*, *N. glutinosa*, *N. tabacum*	*pcna*, *pds*, *su*, *gfp*	[85]
Turnip yellow mosaic virus, RNA virus, monopartite	Tymovirus	Brassicaceae	*A. thaliana*	*pds*, *lfy*	[86]

Table 1.
Plant viruses used as VIGS vectors, the nature of their genomes, and their important hosts.

gene cloning replaced by DNA vector. For defect of viral coat protein production, β genome has been deleted. Every one of the altered DNAs is utilized to blend RNAs by in vitro transcription. As of late, Brome mosaic virus strain has been adjusted for VIGS of pds, actin, and rubisco activase. These genes were additionally silenced in significant model plants, for example, rice. Conventions for VIGS are as follows:

4.1 Target sequence determination

siRNA-Finder (si-Fi; http://labtools.ipk-gatersleben.de/) software that are predicted to produce high numbers of silencing effective siRNAs could be used to select 250–400 nt sequence regions. Whenever the situation allows, select at any rate two preferably non-overlapping regions of the gene of enthusiasm for VIGS investigations. Perception of a similar phenotype initiated by silencing utilizing every one of the at least two free VIGS constructs is a decent sign that the phenotype is because of explicit silencing of the expected target gene, in this manner permitting more noteworthy trust in the acquired outcomes. When endeavoring to silence, an individual from a gene family considers choosing the sequences from the

30 or 50 UTR regions, which are commonly more factor than the CDS. This ought to limit the danger of off-target silencing. Then again, in cases among different gene family members, when a great deal of functional redundancy is expected, it should be possible to design VIGS construct(s) from the conserved gene regions in order to target several or even all gene family members simultaneously. Regarding VIGS experimental design, VIGS construct containing a 250–400 nt fragment of a non-plant origin gene, such as the *Aequorea victoria* Green Fluorescent Protein gene or the *Escherichia coli* β-glucuronidase gene, should be included as at least one negative control.

4.2 VIGS construct formation

Clone the VIGS target sequences into the, for instance, BSMV RNAc vector pCa-cbLIC by means of ligation-independent cloning (LIC), in either sense or antisense direction. Antisense constructs might be progressively effective in initiating gene silencing. Changed sequence checked pCa-cbLIC VIGS construct into *A. tumefaciens* GV3101 by electroporation. For this, MicroPulser (Bio-Rad) electroporator, 0.1 cm gap electroporation cuvettes, and home-made electro-competent cells could be utilized. *Agrobacterium* cultures developed to a last OD600 of 1.2, and the cells will be pelleted by centrifugation and washed in ice-cold sterile 10% glycerol in complete multiple times. Electroporation should be possible, utilizing the producer's pre-set conditions for *Agrobacterium*, for example, one 2.2 kV pulse. Plate an aliquot of the transformation mixture on LB agar enhanced with 25 μg/ml gentamycin and 50 μg/ml kanamycin. As BSMV requires every one of the three genomic fragments, RNAa, RNAb, and RNAc, for effective infection, it is additionally important to deliver *A. tumefaciens* GV3101 strains containing pCaBS-α (BSMV RNAα) and pCaBS-β (BSMV RNAβ).

4.3 Preparation of virus inoculum and infecting target plants with engineered virus

Engineered virus introduced into the leaf of dicot plants (for example well studied *Nicotiana benthamiana*) by means of agroinfiltration. For *N. benthamiana* agroinfiltration, grow 5 ml cultures (LB enhanced with 25 μg/ml gentamycin and 50 μg/ml kanamycin) of *A. tumefaciens* strains conveying pCa-cbLIC VIGS constructs overnight at 28°C with steady shaking at 220 rpm. For each BSMV RNAc build, BSMV RNAα and RNAβ developed in 5 ml cultures will likewise be required. Pellet the *A. tumefaciens* cells at 2500 rcf for 20 min, resuspend it in infiltration buffer [10 mM MgCl2, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES) pH 5.6, and 150 μM acetosyringone] to a last optical density at 600 nm (OD600), and incubate it at room temperature without shaking for 3 h or more. Blend *A. tumefaciens* strains conveying BSMV RNAα, RNAβ, and RNAγ strains together in a 1:1:1 proportion, and pressure infiltrate the bacteria into the abaxial side of completely extended leaves of roughly 25–30-day-old *N. benthamiana* plants utilizing a needleless 1 ml syringe. Utilize 0.5–1 ml of *Agrobacterium* suspension per leaf and mean to penetrate the entire zone of each leaf.

4.4 Assessment of virus-induced gene silencing

Quantitative reverse transcription PCR (qRT-PCR) is used for the assessment of successful silencing of the target gene in the VIGS construct-infected plants. The primers utilized for this reason should tie outside the region focused for silencing.

4.5 Viral infection to the plant and disease evaluation

In the wake of affirming the killing of target gene, one needs to infect the host (plant) from the susceptible virus for the disease appraisal. Genes were focused on that delivered unmistakable phenotypes, for example, silencing of GFP in transgenic tobacco communicating GFP, the photobleaching of leaves brought about by lost carotenoid pigments when phytoene desaturase (pds) was disturbed [56]. Different models focused on the chlorophyll biosynthetic enzyme, bringing about plant chlorosis [59], and the cellulose synthase gene, bringing about a change of plant cell dividers. With the underlying accomplishment of VIGS, specialists started focusing on basic genes [60], for example, those engaged with plant resistance [60] encoding metabolic enzymes, expanding crop yield, or plant development and advancement. For instance, when a VIGS vector developed with tobacco rattle virus was adjusted with the EDS1 gene required for N-intervened resistance from TMV, the immunized plants had an improved susceptibility to TRV.

5. Next-generation VIGS with CRISPR/Cas system

In plant biology, virus-induced gene silencing has made a tremendous impact by silencing and then identifying endogenous genes. However, it is now possible for targeted genome editing and precise knocking out of entire genes with one of the most recent and promising genetic tools, the CRISPR/Cas DNA system. In ongoing investigations, CRISPR/Cas9 was utilized to alter plant genomes, for example, rice, *N. benthamiana* and *Arabidopsis* for heritable changes. The method is basic and requires just transgenic plants expressing cas9 and guide RNA. (The specialized terms are clarified beneath.) Moreover, the hereditary changes are available in ensuing ages. The VIGS system, other than its capacity to silence genes, has discovered a significant application in the CRISPR/Cas altering system. It very well may be utilized as a vehicle to ship the CRISPR/Cas altering system into plant system.

In spite of the fact that this innovation is new, various evidence of idea concentrates in model plants have indicated its potential as a gene editing technology. The productivity, precision, and adaptability of the CRISPR/Cas9 genome engineering system have been exhibited in different eukaryotes, for example, yeast, zebrafish, and worms. The potential applications have been developing quickly which incorporate the forefront use of gene altering in the germlines of people and different life forms. This technique was as of late received in plant systems in different transient tests or in transgenic plants and is turning into the strategy for decision for plant researchers.

Like RNA interference, the CRISPR/Cas gene altering innovation was gotten from a normally happening plant protection mechanism. It gives a type of acquired immunity to the cleavage of DNA present in specific prokaryotes and gives obstruction against foreign hereditary components, for example, phages and plasmids. It depends on the type II clustered regulatory interspaced short palindromic repeats (CRISPR). CRISPR is a sequence of short, monotonous portions followed by a short fragment of spacer DNA. The spacer DNA could be from past exposures to a virus, plasmid, or bacterium. A proof that the wellspring of the spacers was a bacterial genome was the principal trace of the CRISPR's job in an adaptive immunity equivalent to RNA interference. It was before long recommended that the spacers recognized in bacterial genomes filled in as templates for RNA molecules that the bacteria transcribed following a presentation to an attacking phage. Further examinations uncovered that a significant protein called Cas9 was included, together with the transcribed RNA, to perceive the attacking phage and cut the RNA into small

pieces (crRNA) in the CRISPR system. CRISPRs are found in practically 90% of the sequenced Archaea and up to 40% of bacterial genomes. Local bacterial CRISPR RNAs likewise can be adjusted into a single gene known as a single guide RNA (sgRNA). Utilizing sgRNA, the system has gotten increasingly adaptable, permitting it to streamline genome altering by consolidating sgRNA and Cas9 out of a heterologous framework. In plants, the CRISPR/Cas9 system utilizes the two segments; the Cas9 compound catalyzes DNA cleavage and the sgRNA initiates Cas9 to the objective site. This site is generally situated around 20 nucleotides before the protospacer theme and cuts the DNA. Plants utilize the natural mechanism, to reattach the cleaved ends of DNA called nonhomologous end joining [37] and typically bring about a change either by frameshift, addition/erasure, or inclusion of a stop codon. Hence, by essentially designing a sgRNA with a corresponding sequence, for all intents and purposes, any gene can be altered with this heterologous system.

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References

[1] Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. The Plant Cell. 1990;**2**(4):279-289

[2] Ecker JR, Davis RW. Inhibition of gene expression in plant cells by expression of antisense RNA. Proceedings of the National Academy of Sciences of the United States of America. 1986;**83**(15):5372-5376

[3] Romano N, Macino G. Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Molecular Microbiology. 1992;**6**(22):3343-3353

[4] Van Blokland R, Vander Geest N, Mol JNM, Kooter JM. Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. The Plant Journal. 1994;**6**(6):861-877

[5] Covey S, Al-Kaff N, Lángara A, Turner D. Plants combat infection by gene silencing. Nature. 1997;**385**(6619):781-782

[6] Ratcliff F, Harrison B, Baulcombe D. A similarity between viral defense and gene silencing in plants. Science. 1997;**276**(5318):1558-1560

[7] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;**391**(6669):806-811

[8] Guo S, Kemphues K. par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell. 1995;**81**(4):611-620

[9] Pal-Bhadra M, Bhadra U, Birchler J. Cosuppression in *Drosophila*: gene silencing of alcohol dehydrogenase by white-Adh transgenes is polycomb dependent. Cell. 1997;**90**(3):479-490

[10] Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, et al. The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell. 1999;**99**(2):123-132

[11] Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science. 1999;**286**(5441):950-952

[12] Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature. 2000;**404**(6775):293-296

[13] Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 2000;**101**(1):25-33

[14] Tomari Y, Zamore PD. Perspective: Machines for RNAi. Genes & Development. 2005;**19**:517-529

[15] Meister G, Landthaler M, Patkaniowska A, Yair D, Teng G, Tuschl T. Human argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Molecular Cell. 2004;**15**(2):185-197

[16] Cenik ES, Fukunaga R, Lu G, Dutcher R, Wang Y, Tanaka Hall TM, et al. Phosphate and R2D2 restrict the substrate specificity of Dicer-2, an ATP-driven ribonuclease. Molecular Cell. 2011;**42**(2):172-184

[17] Zhang H, Kolb F, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. Cell. 2004;**118**:57-68

[18] Macrae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, et al. Structural basis for double-stranded RNA processing by Dicer. Science. 2006;**311**(5758):195-198

[19] Höck J, Weinmann L, Ender C, Rüdel S, Kremmer E, Raabe M, et al. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. EMBO Reports. 2007;**8**(11):1052-1060

[20] Martinez J, Patkaniowska A, Urlaub H, Lührmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 2002;**110**(5):563-574

[21] Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. A dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. Cell. 2004;**117**(1):83-94

[22] Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005;**123**:631-640

[23] Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, et al. Purified Argonaute 2 and an siRNA form recombinant human RISC. Nature Structural & Molecular Biology. 2005;**12**(4):340-349

[24] Vaucheret H. Plant argonautes. Trends in Plant Science. 2008;**13**(7):350-358

[25] Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, et al. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell. 2001;**106**(1):23-34

[26] Hutvagner G, Simard MJ. Argonaute proteins: Key players in RNA silencing. Nature Reviews. Molecular Cell Biology. 2008;**9**(1):22-32

[27] Yigit E, Batista PJ, Bei Y, Pang KM, Chen CC, Tolia NH, et al. Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell. 2006;**127**:747-757

[28] Meins F, Si-Ammour A, Blevins T. RNA silencing systems and their relevance to plant development. Annual Review of Cell and Developmental Biology. 2005;**21**(1):297-318

[29] Aravin AA, Hannon GJ, Brennecke J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science. 2007;**318**:761-764

[30] Brennecke J, Malone CD, Aravin AA, Sachidanandam R, Stark A, Hannon GJ. An epigenetic role for maternally inherited piRNAs in transposon silencing. Science. 2008;**322**:1387-1392

[31] Tang G. siRNA and miRNA: An insight into RISCs. Trends in Biochemical Sciences. 2005;**30**(2):106-114

[32] Richard WC, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. Cell. 2009;**136**(4):642-655

[33] Filipowicz W, Jaskiewicz L, Kolb FA, Pillai SR. Post-transcriptional gene silencing by siRNAs and miRNAs. Current Opinion in Structural Biology. 2005;**15**:331-341

[34] Ranjan T, Kumari N, Sahni S, Prasad BD. RNA interference: A versatile tool for functional genomics and unraveling the genes required for viral disease resistance in plants. Current Journal of Applied Science and Technology. 2019;**35**(5):1-20

[35] Hannon GJ, Rossi JJ. Unlocking the potential of the human genome

with RNA interference. Nature. 2004;**431**(7006):371-378

[36] Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;**303**(5654):95-98

[37] Hammond SM. Dicing and slicing: The core machinery of the RNA interference pathway. FEBS Letters. 2005;**579**(26):5822-5829

[38] Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, et al. Genetic and functional diversification of small RNA pathways in plants. PLoS Biology. 2004;**2**(5):E104

[39] Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell. 2003;**115**:209-216

[40] Tomari Y, Matranga C, Haley B, Martinez N, Zamore PD. A protein sensor for siRNA asymmetry. Science. 2004;**306**:1377-1380

[41] Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell. 2001;**107**(3):309-321

[42] Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes & Development. 2001;**15**(2):188-200

[43] Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nature Cell Biology. 2005;**7**(7):719-723

[44] Sen GL, Blau HM. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nature Cell Biology. 2005;**7**(6):633-636

[45] Mette MF, Aufsatz W, Vander-Winden J, Matzke MA, Matzke AJ. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. The EMBO Journal. 2000;**19**(19):5194-5201

[46] Wassenegger M, Heimes S, Riedel L, Sänger HL. RNA-directed de novo methylation of genomic sequences in plants. Cell. 1994;**76**(3):567-576

[47] Baulcombe D. RNA silencing in plants. Nature. 2004;**431**:356-363

[48] Vargason JM, Szittya G, Burgyan J, Tanaka HTM. Size selective recognition of siRNA by an RNA silencing suppressor. Cell. 2003;**115**:799-811

[49] Ding SW. RNA-based antiviral immunity. Nature Reviews. Immunology. 2010;**10**:632-644

[50] Silhavy D, Molnár A, Lucioli A, Szittya G, Hornyik C, Tavazza M, et al. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. The EMBO Journal. 2002;**21**:3070-3080

[51] Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, et al. An endogenous, systemic RNAi pathway in plants. The EMBO Journal. 2010;**29**:1699-1712

[52] Chen HY, Yang J, Lin C, Yuan YA. Structural basis for RNA-silencing suppression by Tomato aspermy virus protein 2b. EMBO Reports. 2008;**9**:754-760

[53] Azevedo J, Garcia D, Pontier D, Ohnesorge S, Yu A, Garcia S, et al. Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes & Development. 2010;**24**:904-915

[54] van Kammen A. Virus-induced gene silencing in infected and transgenic

plants. Trends in Plant Science. 1997;**2**:409-411

[55] Baulcombe D. Fast forward genetics based on virusinduced gene silencing. Current Opinion in Plant Biology. 1999;**2**:109-113

[56] Ruiz MT, Voinnet O, Baulcombe DC. Initiation and maintenance of virus-induced gene silencing. The Plant Cell. 1998;**10**:937-946

[57] Voinnet O. RNA silencing as a plant immune system against viruses. Trends in Genetics. 2001;**17**(8):449-459

[58] Brigneti G, Voinnet O, Li WX, Ji L, Ding SW, Baulcombe DC. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. The EMBO Journal. 1998;**17**(22):6739-6746

[59] Kjemtrup S, Sampson KS, Peele CG, Nguyen LV, Conkling MV, Thompson WF, et al. Gene silencing from plant DNA carried by a Geminivirus. The Plant Journal. 1998:91-100

[60] Peele C, Jordan CV, Muangsan N, Turnage M, Egelkrout E, Eagle P, et al. Silencing of a meristematic gene using geminivirus-derived vectors. The Plant Journal. 2001;**24**:357-366

[61] Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, et al. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science. 1986;**232**:738-743

[62] Bucher E, Lohuis D, van Poppel PM, Geerts-Dimitriadou C, Goldbach R, Prins M. Multiple virus resistance at a high frequency using a single transgene construct. The Journal of General Virology. 2006;**87**:3697-3701

[63] Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O. Transitivitydependent and-independent cell-to-cell movement of RNA silencing. The EMBO Journal. 2003;**22**:4523-4533

[64] Schwab R. Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell. 2006;**18**:1121-1133

[65] Fofana IB, Sangare A, Collier R, Taylor C, Fauquet CM. A geminivirus-induced gene silencing system for gene function validation in cassava. Plant Molecular Biology. 2004;**56**:613-624

[66] IIgarashi AK, Kousuke Y, Tomokazu S, Yukari T, Emiko S, Aihiro T, et al. Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, *Arabidopsis thaliana*, cucurbits, and legume. Virology. 2009;**386**(2):407-416

[67] Holzberg S, Brosio P, Gross C, Pogue GP. Barley stripe mosaic virus-induced gene silencing in a monocot plant. The Plant Journal. 2002;**30**(3):315-327

[68] Manmathan H, Shaner D, Snelling J, Tisserat N, Lapitan N. Virus-induced gene silencing of *Arabidopsis thaliana* gene homologues in wheat identifies genes conferring improved drought tolerance. Journal of Experimental Botany. 2013;**64**(5):1381-1392

[69] Atwood SE, O'Rourke JA, Gregory AP, Yin T, Majumder M, Zhang C, et al. Replication protein A subunit 3 and the iron efficiency response in soybean. Plant, Cell and Environment. 2014;**37**:213-234

[70] Zhang C, Ghabrial SA. Development of bean pod mottle virus-based vectors for stable protein expression and sequence-specific

virus-induced gene silencing in soybean. Virology. 2006;**344**(2):401-411

[71] Ding XS, Schneider WL, Chaluvadi SR, Rouf Mian MA, Nelson RS. Characterization of a brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. The American Phytopathological Society MPMI. 2006;**19**(11):1229-1239

[72] Turnage MA, Muangsan N, Peele CG, Robertson D. Geminivirus-based vectors for gene silencing in *Arabidopsis*. The Plant Journal. 2002;**30**(1):107-114

[73] Nagamatsu A, Masuta C, Senda M, Matsuura H, Kasai A, Hong JS, et al. Functional analysis of soybean genes involved in flavonoid biosynthesis by virus-induced gene silencing. Plant Biotechnology Journal. 2007;**5**:778-779

[74] Constantin GD, Krath BN, MacFarlane SA, Nicolaisen M, Johansen IE, Lund OS. Virus-induced gene silencing as a tool for functional genomics in a legume species. The Plant Journal. 2004;**40**:622-631

[75] Naylor M, Reeves J, Cooper JI, Edwards ML, Wang H. Construction and properties of a gene-silencing vector based on poplar mosaic virus (genus *Carlavirus*). Journal of Virological Methods. 2005;**124**(1-2):27-36

[76] Ruiz MT, Voinnet O, Baulcombe DC. Initiation and maintenance of virus-induced gene silencing. The Plant Cell. 1998;**10**:937-946

[77] Gossele V, Fache I, Meulewaeter F, Cornelissen M, Metzlaff M. SVISS – A novel transient gene silencing system for gene function discovery and validation in tobacco plants. The Plant Journal. 2002;**32**:859-886

[78] Hou H, Oiu W. A novel co-delivery system consisting of a tomato bushy stunt virus and a defective interfering RNA for studying gene silencing. Journal of Virological Methods. 2003;**111**(1):37-42

[79] Huang YH, Baoa Y, Penga W, Goldbergb M, Loveb K, Bumcrotc DA, et al. Claudin-3 gene silencing with siRNA suppresses ovarian tumor growth and metastasis. PNAS. 2009;**106**(9):3426-3430

[80] Kumagai MH, Donson J, Della-Cioppa G, Harvey D, Hanley K, Grill AND, et al. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**:1679-1683

[81] Liu Y, Schiff M, Dinesh-Kumar SP. Virus-induced gene silencing in tomato. The Plant Journal. 2002;**31**(6):777-786

[82] Ratcliff F, Martin-Hernandez AM, Baulcombe DC. Tobacco rattle virus as a vector for analysis of gene function by silencing. The Plant Journal. 2001;**25**(2):237-245

[83] Senthil-Kumar M, Udaykumar M. High-throughput virus-induced gene-silencing approach to assess the functional relevance of a moisture stress-induced cDNA homologous to lea4. Journal of Experimental Botany. 2006;**57**(10):2291-2302

[84] Peele C, Jordan CV, Muangsan N, Turnage M, Egelkrout E, Eagle P, et al. Silencing of a meristematic gene using geminivirus-derived vectors. The Plant Journal. 2001;**27**(4):357-366

[85] Tao X, Zhou X, Cui X, Qian Y. Virus induced gene silencing and its application for analysis of genomic function in plants. Europe PMC. 2004;**31**(9):777-783

[86] Pflieger S, Blanchet S, Camborde L, Drugeon G, Rousseau A, Noizet M, et al. Efficient virus-induced gene silencing in *Arabidopsis* using a 'one-step' TYMV-derived vector. The Plant Journal. 2008;**56**:678-690

[87] Tao X, Zhou X. A modified viral satellite DNA that suppresses gene expression in plants. The Plant Journal. 2004;**38**(5):850-860