***Review Article***

# RNA Interference: A versatile tool for Functional Genomics and unraveling the genes required for viral disease resistance in plants

**Abstract:**

Virus-induced gene silencing (VIGS) is a powerful reverse genetics technology used to unravel the functions of genes. It uses viruses as vectors to carry targeted plant genes. The virus vector is used to induce RNA-mediated silencing of a gene or genes in the host plant. The process of silencing is triggered by dsRNA molecules, the mechanism ~~of which~~ is explained in this chapter. Over the years a large number of viruses have been modified for use as VIGS vectors and a list of these vectors is also included. As the name suggests, virus-induced gene silencing uses the host plant's natural defense mechanisms against viral infection to silence plant genes. VIGS is methodologically simple and is widely used to determine gene functions, including disease resistance, abiotic stress, biosynthesis of secondary metabolites and signal transduction pathways. Here, we made an attempt to describe the basic underlying molecular mechanism of VIGS, the methodology and various experimental requirements, ~~and~~ as well as its advantages and disadvantages. Finally, we ~~will consider~~discuss the future prospects of VIGS in relation to CRISPR/Cas9 technology. Besides using it to overexpress or silence genes, VIGS has emerged as the preferred delivery system for the cutting edge CRISPR/Cas9 genome editing technology.

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

**Introduction:**

RNA interference (RNAi) has revolutionized the studies to determine the role of a particular gene. RNAi ~~interference (RNAi)~~ is a biological process where RNA molecule inhibits the expression of a particular gene by targeting and destructing ~~of~~ specific mRNA molecules. RNAi is also known as post-transcriptional gene silencing (PTGS), co-suppression and quelling. The discovery of RNAi was totally serendipity. 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 conducted by Napoli et al. (1990). These plant scientists were trying to introduce additional copies of chalcone synthase gene responsible for darker pigmentation of flowers;~~.~~ t~~T~~he transgenic copy, intended to make more corresponding gene products. But instead of darker flowers, white or less pigmented flowers were observed indicating the suppressed/decreased expression of endogenous chalcone synthase gene (Napoli et al., 1990; Ecker and Davis, 1986). This suggested~~s~~ down regulation of endogenous gene by the event post-transcriptional inhibition due to their mRNA degradation (Romano and Macino, 1992, Van Blokland et al., 1994). Silencing of target genes by RNA interference technology came in to the lime light just after discovery of plant defense mechanism against virus, where it was believed that plant encode short, non-coding region of viral RNA sequences, which after infection recognize and degrades viral mRNA. These short and non-coding RNA sequences might be against viral DNA/RNA polymerase and other important genes necessary for viral infection and multiplication. On the theme of above concept plant virologists introduced short nucleotide~~s~~ sequences into the viruses and expression of target genes in the infected plants was ~~found to be~~ suppressed (Covey et al., 1997; Ratcliff et al., 1997). This most popular phenomenon is known as 'virus-induced gene silencing' and brings the boom in the era of biotechnologists. Just after a year later in 1998,

Craig Mello and Andrew Fire~~'s performed worked in the laboratory to study~~studied the effect of RNAi in *C. elegans* and interestingly they found that dsRNA effectively silenced the target gene in comparison to antisense ssRNA (100 folds more potent).

The term RNAi was coined by these two scientists for the first time and they were awarded Nobel Prize in the field of medicine in 2006 for this breakthrough (Fire et al., 1998). After this great discovery of dsRNA as an extremely potent trigger for gene silencing, it became very realistic to unravel the mechanism of RNAi action in various biological systems (Guo and Kemphues, 1995; Pal-Bhadra et al., 1997).

Protein~~s~~ machinery necessary for gene silencing was discovered in *C. elegans* for the first time in 1999 and comprehensive analysis indicated~~s~~ that common fundamental mechanism must be operated throughout the eukaryotes such as fungi, *Drosophila* and plants (Tabara et al., 1999). The s~~S~~cientific community ~~has~~ started to realize~~ing~~ that the RNAi pathway has an ancient origin, ~~and~~ coming from primitive eukaryotes to recent human beings. ~~Paralelly in the same meanwhile~~At the same time, different groups of scientists working on PTGS system in plants, *Drosophila* and worm came up with interesting facts and their results were ~~par~~ consistent with each other. They observed that small RNA ranging in length from 21-23 nucleotides generated from dsRNA in cell extracts, and could serve as a *de novo* silencing trigger for RNAi in cell extracts free of dsRNA treatments. They concluded that short 21-23 nucleotides siRNAs ~~are~~ were the outcome of Dicer and RNA-induced silencing complex (RISC) (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). Now these days, engineered synthetic RNAs ha~~s~~ves been extensively used to induce sequence~~--~~specific gene silencing and became a very popular tool for knocking down ~~of~~ eukaryotic genes. As with many great

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discoveries, the history of RNAi is a tale of scientists able to interpret unexpected results in a novel and imaginative way.

The short RNA molecules, a key to RNA interference technology are of two types; (I) microRNA (miRNA) and (II) small interfering RNA (siRNA). miRNAs are endogenous or purposefully expressed product (organism own genome product), whereas siRNAs are derived product of exogenous origin such as virus, transposon. Both have different precursors for example miRNA seems to be processed from stem-loop with partial complementary dsRNA whereas siRNA appears from fully complementary dsRNA (Tomari and Zamore, 2005). ~~Inspite~~ Despite of these differences, both short nucleotides are very much related in terms of their biogenesis and mode of action (Meister and Tuschl, 2004). Like, both Dicer and RISC assembly is needed during their synthesis from precursor molecules and targeting as well. Small RNAs are the key mediators of RNA silencing and related pathways in plants and other eukaryotic organisms. Silencing pathways couple the destruction of double-stranded RNA with the use of the resulting small RNAs to target other nucleic acid molecules that contain the complementary sequence. This discovery has revolutionized our ideas about host defense and genetic regulatory mechanisms in eukaryotes. Small RNAs can direct the degradation of mRNAs and single-stranded viral RNAs, the modification of DNA and histones, and the inhibition of translation. Viruses might even use small RNAs to do some targeting of their own to manipulate host gene expression.

## PRINCIPAL COMPONENTS LIES AT THE HEART OF ~~RNAI~~ RNAi PATHWAY

### Dicer: A Gateway into the RNA interference

Dicer, a member of RNase III family proteins with dsRNA-specific nuclease activity, ~~and it~~ acts as a primary candidate for biogenesis of siRNA during gene silencing (Tomari and Zamore, 2005). These enzymes have several critical motifs spread throughout the polypeptide chain from N-terminus to C-terminus, which are~~is~~ responsible for ~~their~~ efficient performance (Meister and Tuschl, 2004). RNase III enzymes are~~is~~ characterized by the domains in order from N-to-C terminus: a DEXD domain, a DUF283 domain, a PAZ (Piwi/Argonaute/Zwille) domain, two tandem RNaseIII domain and a dsRNA binding domain (Figure 1A). Apart from ribonuclease-specific PAZ domain, Dicer do possess helicase domain and ~~their~~ its function has been implicated in processing long dsRNA substrate (Cenik et al., 2011). Out of these five crucial domains, PAZ and RNase III are very critical for precise excision of siRNA from dsRNA precursor (Zhang et al., 2004) (Figure 1B). PAZ domain recognizes the duplex RNA end with three-~~-~~nucleotide~~s~~ overhang, resulting in stretching of two helical turns along the surface of the protein. This leads to the cleavage of one out of the two strands at a time by two different RNase III domains separately. The final product after Dicer action is 21-23 nt long fragments with two-nucleotide~~s~~ overhang at the 3' end~~-~~, which now act as a substrate for RISC (Tomari and Zamore, 2005). Current findings suggests that PAZ domain is capable of binding the exactly 2 nucleotide 3' overhang of dsRNA while the RNaseIII catalytic domains form a pseudo dimer around the dsRNA to initiate cleavage of the strands. This results in a functional shortening of the dsRNA strand. The distance between the PAZ and RNaseIII domains is determined by the angle of the connector helix and influences the length of the micro RNA product (Macrae et al., 2006). In some ~~of the~~ organism, only one copy of Dicer is responsible for the processing of both miRNA and siRNA but interestingly, in *Drosophila* Dicer 1 is solely devoted for miRNA biogenesis while Dicer 2 used for siRNA track (Tomari and Zamore, 2005).

The molecular weight of Dicer ranges from 80kDa to 219kDa (Human Dicer). The difference in size is due to the presence of all five domains in human Dicer and absence of few domains in Dicer characterized from *Giardia intestinalis*. Other variants of Dicer are characterized by 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~~on substrate molecules, ~~but study also give clue~~it has been shown that ATPase domain is ~~very~~ critical for switching/moving~~ement~~ of both RNase III domains, and biochemical studies indicate that~~s~~ mutations in the ATPase domain lead~~s~~ to the abolishment of siRNAs ~~procession~~ production (Tomari and Zamore, 2005). Because most vertebrates, especially *C. elegans*, express only one Dicer protein, interactions with additional proteins must modulate the specificity of these enzymes. A s~~S~~tudy indicates that the R2D2-like protein, RDE-1 & 4, form a complex with Dicer and is essential for the RNAi pathway but not for miRNA functioning (Tabara et al., 2002).

## RISC: At the Core 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 general, RISC programming is triggered by the appearance of dsRNA in the cytoplasm of a eukaryotic cell. RISC is a multiprotein complex composed of ribo-nucleoproteins (Argonaute protein), incorporates one strand of dsRNA fragments (siRNA, miRNA) to the target transcripts. To purify RISC, Tuschl and colleagues used cell extracts derived from human HeLa cells. They partially purified~~y~~ RISC by conjugating the 3' termini of siRNAs to biotin, which enabled co-immunoprecipation of the siRNA with associated protein complexes. Precipitated complexes were further purified based on size and molecular weight. Two proteins of ~100 kDa were also identified that corresponded to Argonaute 1 and Argonaute 2 (Ago1 and Ago2). Biochemical isolation~~s~~ of RISC ~~have~~ revealed a

variety of different RNPs, ranging from modest size (150 kDa) up to 3 MDa particle termed 'holo-RISC' and many other intermediate sizes haves also been observed (Hock et al., 2007; Martinez et al., 2002; Pham et al., 2004). The complete structure of RISC is still unsolved. Recent research has reported a large number of RISC-associated proteins, which includes mainly, Argonaute proteins and RISC-loading complex. These both 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) (Figure 1E).

In 2005, Gregory et al. identified a 500 kDa minimal RISC by characterizing proteins that copurified with human Dicer. Two proteins were found to be associated with Dicer, Ago2, and TRBP (the HIV trans-activating response RNA-binding protein) (Gregory et al., 2005). ParalellyAt the same time, the minimal RISC, sufficient for target RNA recognition and cleavage efficiently, was demonstrated to be simply an Argonaute protein bound to a small RNA (Rivas et al., 2005). Argonaute proteins are ubiquitously found in plant, animal, many fungi, protista and even in few archaea as well. Although all AGO proteins harbour PAZ, MID (middle) and PIWI domains, they are divided into three groups on the basis of both their phylogenetic relationships and their capacity to bind to small RNAs. Group 1 members bind to microRNAs (miRNAs) and small interfering RNAs (siRNAs) and are referred to as AGO proteins. Group 2 members bind to PIWI-interacting RNAs (piRNAs) and are referred to as PIWI proteins. Group 3 members have been described only in worms, where they bind to secondary siRNAs. AGOs are large proteins (ca 90–100 kDa) consisting of one variable N-terminal domain and conserved C-terminal PAZ, MID and PIWI domains. Experiments with bacterial and animal AGO proteins have elucidated the roles of these three domains in small RNA pathways. The MID domain binds to the 5'

phosphate of small RNAs, whereas the PAZ domain recognizes the 3' end of small RNAs. The PIWI domain adopts a folded structure similar to that of RNaseH enzymes and exhibits endonuclease activity, which is carried out by an active site usually carrying an Asp–Asp–His (DDH) motif (Vaucheret, 2008).

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 genes ranging from a single copy to dozens of copies (even more than two dozens) ~~is found to be~~have been observed. Multiple copies (~~Paralogous~~ paralogous proteins) of Argonaute proteins in *C. elegans* reflect~~s~~ their functional~~ly~~ redundancy, ~~and~~ but their evolutionary significance ~~is~~ remains unknown. Some studies suggest that genes encoding for Argonaute proteins ~~ample to recompense~~compensate for one another (Grishok et al., 2001).

The Argonaute associated with siRNA binds to the 3'-untranslated region of mRNA and prevents the production of proteins in several ways. The recruitment of Argonaute proteins to targeted mRNA can induce mRNA degradation. The Argonaute-miRNA complex can also effect the formation of functional ribosomes at the 5'-end of the mRNA. The complex competes with ~~the~~ translation initiation factors and/or abrogates ribosome assembly. Also, the Argonaute-miRNA complex can adjust protein production by recruiting cellular factors such as peptides or post translational modifying enzymes, which degrade the ~~growing of~~ polypeptide growth~~s~~ (Hutvagner and Simard, 2008).

The Argonaute superfamily can be divided into three separate subgroups: the Piwi clade that binds piRNAs, the Ago clade that associates with miRNAs and siRNAs, and a third clade that has only been found and characterized in nematodes so far (Yigit et al., 2006). All gene-regulatory phenomena involving ~20−30 nt RNAs are thought to require one or more Argonaute

proteins, and these proteins are the central, defining components of the various forms of RISC. The double-stranded products of Dicer enter 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 (Meister and Tuschl, 2004; Tomari and Zamore, 2005). Thus one guide strand directs target recognition by Watson-Crick base pairing, whereas the other strand of the original small RNA duplex, known as the passenger strand, is discarded.

In humans, there are eight AGO family members, some of which ~~are~~ have been investigated intensively. However, even though AGO1-4 are~~is~~ capable of loading miRNA~~,~~ and perform endonuclease activity, ~~but~~ RNAi dependent gene silencing is exclusively found with AGO2. Considering the sequence conservation of PAZ and PIWI domains across the family, the uniqueness of AGO2 is presumed to arise from either the N-terminus or the spacing region linking PAZ and PIWI motifs.

Several AGO family members in plants also attract~~s~~ tremendous effort of studying. AGO1 is clearly involved in miRNA--related RNA degradation, and plays a central role in morphogenesis. In some organisms, it is strictly required for epigenetic silencing. Interestingly, it is regulated by miRNAs itself. AGO4 ~~does~~ is not involved in RNAi--directed RNA degradation, but in DNA methylation and other epigenetic regulation mechanisms, through small RNA (siRNA) pathway. AGO10 is involved in plant development. AGO7 has a function distinct from AGO 1 and 10, and is not found in gene silencing induced by transgenes. Instead, it is related to developmental timing in plants (Meister et al., 2004; Meins et al., 2005). At the cellular level, Ago proteins localize diffusely in the cytoplasm and nucleus and, in some cases, also at distinct foci, which include P-bodies and stress granules. The second clade, Piwi (named after the *Drosophila* protein PIWI, for P-element-induced wimpy testis), is most abundantly expressed in germ line cells and

functions in the silencing of germ line transposons. A major biochemical difference between Argonaute clades is the means by which members acquire guide RNAs. Ago guides RNAs ~~which~~that ~~are~~ have been generated from dsRNA in the cytoplasm by a specialized nuclease named Dicer. Members of the Piwi clade are thought to form guide RNAs in a "ping-pong" mechanism in which the target RNA of one Piwi protein is cleaved and becomes the guide RNA of another Piwi protein. Maternally inherited guide piRNAs are believed to initiate this gene-silencing cascade. Class 3 Argonautes obtain guide RNAs by Dicer-mediated cleavage of exogenous and endogenous long dsRNAs (Aravin et al., 2007; Brennecke et al., 2008; Yigit et al., 2006).

The hall mark domains of Argonaute proteins are~~:~~, N-terminal PAZ (similar to Dicer enzymes and share common evolutionary origin), mid domain and C-terminal PIWI domain, a unique to the Argonaute superfamily proteins (Figure 1 C & D). The PAZ domain is named after discovery of proteins PIWI, AGO, and Zwille~~, whereby it is found to conserve~~. The PAZ domain interacts with 3'end of both siRNA/miRNA in a sequence~~-~~-independent manner, and finally it hybridizes with the target mRNA via base-pairing interaction, leadings~~s~~ to ~~the~~ cleavage or translation inhibition (Tang, 2005). PIWI domain, which is very essential for RNA backbone cleavage has a structural~~ly~~ resemblance with RNaseH. The active site is composed of triad amino acids, aspartate-aspartate-glutamate, which co-ordinate~~s~~ with divalent metal ions and provides binding energy for catalysis. In few Argonaute proteins, PIWI domain participates in interaction with the Dicer via one of the RNaseIII domain (Meister et al., 2004). Between the Mid and PIWI domain, a MC motif is present which is thought to be involved in interaction sites for the 5'cap of siRNA/miRNA and control their translation (Hutvagner and Simard, 2008). The overall structure of Argonaute is bilobed, with one lobe consisting of the PAZ domain and the other lobe

consisting of the PIWI domain flanked by N-terminal (N) and middle (Mid) domains (Figure 1 C & D). The Argonaute PAZ domain has RNA 3′ terminus binding activity, and the co-crystal structures reveal that this function is used in guide strand binding. 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. The protein-DNA contacts are dominated by sugar-phosphate backbone interactions, as expected for a protein that can accommodate a wide range of guide sequences. Guide strand nucleotides 2−6, which are especially important for target recognition, are stacked with their Watson-Crick faces exposed and available for base pairing (Richard et al., 2009).

## GENERAL MECHANISM OF RNAi

The RNAi pathway, ubiquitous to most of the eukaryotes, ~~is~~ consists of short RNA molecules that binds to specific target mRNAs, forming a dsRNA hybrid, and inactivating~~e~~ the mRNA by preventing ~~from producing a~~ protein synthesis. Apart from their role in defense against viruses, protozoans, it also influences ~~the~~ development ~~of organisms~~. During RNAi, the dsRNA formed in cells by DNA- or RNA-dependent synthesis of complementary strands, or introduced into cells by viral infection or artificial expression, is processed to 20-bp double-stranded small interfering RNAs (siRNAs) containing 2-nt 3’ overhangs (Filipowicz et al., 2005). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC), which mediates the degradation of mRNAs with sequences fully complementary to the siRNA (Figure 2). In another recent pathway, occurring in the nucleus, siRNAs formed from repeat element transcripts and incorporated into the RNAi-induced transcriptional silencing (RITS) complex may guide chromatin modification and silencing. The genetics and biochemistry of the

latter process are best characterized for ~~the~~ plants and yeast, but related pathways also operate in other organisms (Lippman and Martienssen, 2004).

## Initiation: Processing of Precursor dsRNA

In the RNAi pathway, an RNA-~~-~~dependent pathway can be activated by either exogenous or endogenous short dsRNA molecules in the cytoplasm. The precursor of siRNAs is termed ~~as~~ primary siRNA or pri-siRNA, folds back to form a long stem-loop structure (endogenous source dsRNA), leaving two 3'overhang nucleotides and the 5'phosphate group at the cleavage site (Hannon et al., 2004). In case of miRNA, Drosha and Pasha are responsible for trimming the end of stem-loop like pri-miRNA inside the nucleus, leading to the generation of pre-miRNA. Now, this pre-miRNA is transported to the cytoplasm by the help of Ran-GTP mediated exportin-5 nuclear transporter, where Dicer chops the dsRNA into mature miRNA (Lund et al., 2004).

Processing of exogenous RNAs is cytoplasmic, ~~that leads to~~in this case the biogenesis of siRNAs 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 (PWZ). The PWZ domain is also found in Argonaute family proteins, known to be very essential for RNAi. The current finding suggests the binding of Dicer to the end of dsRNA is far more effective than internal binding. Dicer will associate with an existing terminus of dsRNA ~~and cuts~~cutting ~21 nucleotides away from the end, forming a new end with two 3' overhangs. As a result of this stepwise cutting, a pool of 21-nt long small RNA with two 3' overhang~~s.-~~nucleotides will be generated from long dsRNAs (Hammond, 2005). Several organisms contain more than one Dicer genes, with each Dicer preferentially processing dsRNAs from different sources. *Arabidopsis thaliana* has four Dicer-like proteins. Out of which DLC-1 ~~is~~ participate~~d~~ in microRNA

maturation; DLC-2 preferentially processes dsRNA from plant viruses; DLC-3 is required for generating small RNAs from endogenous repeated ~~-~~sequences. Interestingly, most of the mammals encode only one Dicer gene (Xie et al., 2004).

## 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, miRNAs and siRNAs present in functional RISCs have to be single stranded for pairing with the target RNA. How are the duplexes converted to single-chain forms and how is a correct (i.e. antisense or 'guide') strand selected for loading onto the RISC? The latter question is of practical importance because artificial siRNAs can be directly used to trigger RNAi in order to knock-down genes. Measurements of the potency of different double- and single stranded siRNAs, and sequence analysis of the duplexes formed by pre-siRNA processing by Dicer have indicated that the strand incorporated into ~~the~~ RISC is generally the one whose 5' terminus is the thermodynamically less stable end of the duplex (Khvorova et al., 2003). 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. Photocross-linking to siRNAs containing 5-iodouracils at different positions demonstrated that Dicer binds to a less stable and R2D2 to a more stable siRNA end. The most conserved members of RISC are Argonaute proteins, which are essential mostly for RISC function~~s~~. Argonaute proteins are highly rich in basic amino acids and these residues are ~~basically r~~esponsible for cross-linking with the guide RNA in plants (Tomari et al., 2004).

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 also appears in Dicer proteins, specifically recognizes the unique structure of two 3' nucleotide~~-s~~ overhangs of

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siRNAs. The 5’ phosphate group is recognized by the PIWI domain in Argonaute proteins and therefore required for siRNA to assembly into RISC. sSiRNAs lacking this phosphate group in the 5’ end will be rapidly phosphorylated by an endogenous kinase (Nykanen et al., 2001). Transfer of Dicer--processed dsRNA to RISC is mediated by several unknown proteins. An ATP dependent process is needed to activate RISC, which helps in unwinding of the siRNA duplex, leaving only single strand RNAs joining the active form of RISC. Studies on comparing the stability between functional and non-functional siRNAs indicates that the 5’ antisense region of the functional siRNAs were are less thermodynamically stable than the 5’ sense regions, providing a basis for their selective entry into the RISC. The strand remainingedwithin the RISC functions as a guild to locate targets -mRNA sequences through Watson-Crick base-paring, while the other stand of duplex siRNA is either cleaved or discarded during the loading process. The endonuclease Argonaute 2, the only member of the Argonaute subfamily of proteins with observed catalytic activity in mammalian cells, is responsible for this slicing activity. Cleaved transcripts will undergo subsequent degradation by cellular exonucleases. The guiding strand of the siRNA duplex inside RISC will be intact during this process and therefore permit RISC to function catalytically. This robust cleavage pathway makes it a very attractive method of choice for potential therapeutic applications of RNAi (Elbashir et al., 2001). Whether siRNA-mediated regulation has an impact on initiation, elongation or termination, or whether it acts co-translationally, is still a matter of debate. For example, Human Ago2 binds to m7GTP and thus can compete with eukaryotic translation initiation factor 4E (eIF4E) for binding to the m7GTP-cap structure of mRNA; association of Human Ago2 with eIF6 and large ribosomal subunits also suggests that miRNAs inhibit an early step of translation. However, miRNAs and AGOs are

found associated with polysomes, suggesting that inhibition occurs after initiation, at least in some cases (Vaucheret, 2008).

In plants, the majority of miRNAs hybridize to target mRNA with a near-perfect complementarity, and mediate an endonucleolytic cleavage through a similar, if not identical, mechanism used by the siRNA pathway. While iIn animals, miRNAs interacts only with 3'UTR sequences of mRNA (For ex; *lin-4*) and regulated expression of proteins negatively. The central mismatch between miRNA-mRNA hybridization is believed to be responsible for the lack of RNAi-mediated mRNA cleavage events (i.e. lack of RISC--mediated mRNA degradation). miRNA-mRNA complex associated with Ago proteins finally transfer to processing body (P-body), where mRNA finally degraded by RISC-independent pathway (Liu et al., 2005; Sen and Blau, 2005).

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RNAi--mediated gene silencing of genes is not limited to the posttranscriptional level only. In plants, it has been shown that siRNA can also trigger *de novo* DNA methylation and transcriptional silencing. Recent evidence suggests that siRNAs can inactivate transcription through direct DNA methylation and other types of covalent modifications in the genomes of certain species. Several studies also demonstrated that RNAi machinery in the fission yeast *S .pombe* plays a critical role in formation and maintenance of higher-order chromatin structure and function. It is has been hypothesized that expression of centromeric repeats results in the formation of a dsRNA that is cleaved by Dicer into siRNAs that directing DNA methylation of heterochromatic sites and regulatinges genethe expression of genes (Mette et al., 2000; Wassenegger et al., 1994). Many plant and some animal viruses encode suppressors of post-transcriptional RNA silencing 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 (Baulcombe, 2004; Vargason et al., 2003).

RNA silencing functions as a natural immunity mechanism in plant defense against pathogen invasion (Ding, 2010), and many viruses have evolved to express virus silencing repressor proteins to counteract host antiviral RNA silencing ~~and~~ mentioned in figure 2.~~–~~ Some of the virus~~-~~ ~~–~~silencing repressors ~~were~~ have been studied at the molecular level, such as 2b of Cucumber mosaic, P69 of the turnip yellow mosaic virus (TYMV), and HC-Pro of the turnip mosaic virus (TuMV), in *Arabidopsis*. The P19 protein of tombusviruses, undoubtedly the best known virus silencing repressor (VSR) so far, prevents RNA silencing by siRNA sequestration through binding ds siRNA with a high affinity (Silhavy et al., 2010). Crystallographic studies have revealed that P19 forms ~~is~~ a tail-to-tail homodimer, which acts like a molecular calliper, measuring the length of siRNA duplexes and binding them in a sequence-independent way, selecting for the 19 bp long dsRNA region of the typical siRNA (Vargason et al., 2003). Latest findings have also confirmed that P19 inhibits the spread of the ds siRNA duplex identified as the signal of RNA silencing (Dunoyer et al., 2010).

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Other VSRs, such as the Tomato aspermy cucumovirus 2b protein or B2 of the insect-infecting Flock house virus, also bind ds siRNA in a size-specific manner; nevertheless, structural studies have shown that their modes of binding siRNAs do not share any similarity with P19 (Chen et al., 2008).

~~Identified t~~Two viral proteins ~~were~~ have been shown to inhibit the processing of dsRNA to siRNAs in agroinfiltration assays: P14 of Pothos latent aureusvirus and P38 of Turnip crinkle virus (TCV). Recently, it was discovered that the action of the P38 protein occurs through

AGO1 binding and that it interferes with the AGO1-dependent homeostatic network, which leads to the inhibition of *Arabidopsis* DCLs (Azevedo et al., 2010). In addition to P14 and P38, the P6 VSR of the Cauliflower mosaic virus (CaMV) [Love et al., 2007] has been shown to interfere with vsiRNA processing. P6 was previously described as a viral translational trans-activator protein essential for virus biology. Importantly, P6 has two importin-alpha dependent nuclear localization signals, which are mandatory for CaMV infectivity. A recent discovery showed that one of the nuclear functions of P6 is to suppress RNA silencing by interacting with dsRNA-binding protein 4, which is required for the functioning of DCL4.

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## VIRUS-INDUCED GENE SILENCING: MECHANISMS AND APPLICATIONS

Van Kammen was the first~~;~~ to use the term 'virus-induced gene silencing' (VIGS) to describe the phenomenon of recovery from virus infection (van Kammen, 1997)~~.~~ ~~t~~Though, the term has since been applied almost exclusively to the technique involving recombinant viruses to knock-~~–~~down expression of endogenous genes (Baulcombe, 1999; Ruiz et al., 1998). RNA silencing has become a major focus of molecular biology and biomedical research around the world. To reduce the losses caused by plant pathogens, plant biologists have adopted numerous methods to engineer resistant plants. Among them, RNA silencing-based resistance has been a powerful tool that has been used to engineer resistant crops during the last two decades. Based on this mechanism, diverse approaches were developed. Virus-induced gene silencing (VIGS) is a virus vector technology that exploits an RNA-mediated antiviral defense mechanism. In plants infected with unmodified viruses the mechanism is specifically targeted 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 used widely in plants for

analysis of gene function and has been adapted for high-throughput functional genomics. Until now most applications of VIGS have been studied in *Nicotiana benthamiana*. However, new vector systems and methods are being developed that could be used in other plants, including *Arabidopsis*. VIGS also helps in the identification of genes required for disease resistance in plants. These methods and the underlying general principles also apply when VIGS is used in the analysis of other aspects of plant biology.

When a plant virus infects a host cell it activates an RNA-based defense that is targeted against the viral genome. The dsRNA in virus-infected cells is thought to be the replication intermediate that causes the siRNA/RNase complex to target the viral single-stranded RNA. In the initially infected cell, the viral ssRNA would not be a target of the siRNA/RNase complex because this replication intermediate would not have accumulated to a high level. However, in the later stages of the infection, as the rate of viral RNA replication increases, the viral dsRNA and siRNA would become more abundant. Eventually, the viral ssRNA would be targeted intensively and virus accumulation would slow down (Voinnet, 2001). Many plant viruses encode proteins that are suppressors of this RNA silencing process. These suppressor proteins would not be produced until after the virus had started to replicate in the infected cell so they would not cause complete suppression of the RNA--based defense mechanism. However, these proteins would influence the final steady-state level of virus accumulation. Strong suppressors would allow virus accumulation to be prolonged and at a high level. Conversely, if a virus accumulates at a low level it could be due to weak suppressor activity (Brigneti et al., 1998). The dsRNA replication intermediate would be processed so that the siRNA in the infected cell would correspond to parts of the viral vector genome, including any non-viral insert. Thus, if the insert is from a host gene, the siRNAs would target the RNase complex to the corresponding host

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mRNA and the symptoms in the infected plant would reflect the loss of the function of the encoded protein.

There are several examples that strongly support this approach to suppression of gene expression. Thus, when tobacco mosaic virus (TMV) or potato virus X (PVX) vectors were modified to carry inserts from the plant phytoene desaturase gene, the photobleaching symptoms on the infected plant reflected the absence of photoprotective carotenoid pigments that require phytoene desaturase. Similarly when the virus carried inserts of a chlorophyll biosynthetic enzyme there were chlorotic symptoms and, with a cellulose synthase insert, the infected plant had modified cell walls (Kjemtrup et al., 1998). Genes other than those encoding metabolic enzymes can also be targeted by VIGS. For example, if the viral insert corresponds to genes required for disease resistance, the plant exhibits enhanced pathogen susceptibility. In one such example the insert in a tobacco rattle virus (TRV) vector was from a gene (EDS1) that is required for N-mediated resistance to TMV. The virus vector-infected N-genotype plant exhibited compromised TMV resistance. The symptoms of a TRV vector carrying a *leafy* insert demonstrated how VIGS can be used to target genes that regulate development. *Leafy* is a gene required for flower development. Loss-of-function *leafy* mutants produce modified flowers that are phenocopied in the TRV-leafy-infected plants. Similarly the effects of tomato golden mosaic virus vectors carrying parts of the gene for a cofactor of DNA polymerase illustrate how VIGS can be used to target essential genes. The plants infected with this geminivirus vector were suppressed for division growth in and around meristematic zones of the shoot (Peele et al., 2001).

To exploit the ability to knock down, in essence, any gene of interest, RNAi via siRNAs has generated a great deal of interest in both basic and applied biology. There are an increasing

number of large-scale RNAi screens that are designed to identify the important genes in various biological pathways. Because disease processes also depend on the combined activity of multiple genes, it is expected that turning off the activity of a gene with specific siRNA could produce a therapeutic benefit to mankind. Based on the siRNAs-mediated RNA silencing (RNAi) mechanism, several transgenic plants haves been designed to trigger RNA silencing by targeting pathogen genomes. Diverse targeting approaches have been developed based on the difference in precursor RNA for siRNA production, including sense/antisense RNA, small/long hairpin RNA and artificial miRNA precursors. Virologists haves been designed many transgenic plants expressing viral coat protein (CP), movement protein (MP) and replication associated proteins, showing resistant against infection by the homologous virus. This type of pathogen-derived resistance (PDR) has been reported in diverse viruses including tobamo-, potex-, cucumo-, tobra-, Carla-, poty-, and alfalfa mosaic virus groups as well as the luteovirus group (Abel et al., 1986; Ding, 2010).

Transgene RNA silencing-mediated resistance is a process that is highly associated with the accumulation of viral transgene-derived siRNAs. One of the drawbacks of the sense/antisense transgene approach is that the resistance is unstable, and the mechanism often results in delayed resistance or low efficacy/resistance. This may be due to the low accumulations of transgene-derived siRNA in PTGS due to defense mechanism encoded by plants. Moreover, numerous viruses, including potyviruses, cucumoviruses, and tobamoviruses, are able to counteract these mechanisms by inhibiting this type of PTGS. Therefore, the abundant expression of the dsRNA to trigger efficient RNA silencing becomes crucial for effective resistance. To achieve resistance, inverse repeat sequences from viral genomes were have been widely used to form hairpin dsRNA in vivo, including small hairpin RNA (shRNA), self-

complementary hpRNA, and intron-spliced hpRNA. Among these methods, self-complementary hairpin RNAs separated by an intron likely elicit PTGS with the highest efficiency. The presence of inverted repeats of dsRNA-induced PTGS (IR-PTGS) in plants also showed high resistance against viruses. IRPTGS is not required for the formation of dsRNA for the processing of primary siRNAs, but the plant RDRs are responsible for the generation of secondary siRNAs derived from non-transgene viral genome, which further intensify the efficacy of RNA silencing induced by hpRNA, a process named RNA silencing transitivity. Among them, the sequence similarity between the transgene sequence and the challenging virus sequence is the most important. Scientists haves engineered several transgenic plants with multiple hpRNA constructs from different viral sources, or with a single hpRNA construct combining different viral sequences, was created. Thus, multiple viruses can be simultaneously targeted, and the resulting transgenic plants show a broader resistance with high efficacy. In addition to the sequence similarity, the length of the transgene sequence also contributes to high resistance. In general, an average length of 100 to 800 nt of transgene sequence confers effective resistance (Bucher et al., 2006; Himber et al., 2003).

Comment [A2]: Please indicate what RDRs stand for.

By mimicking the intact secondary structure or hairpin loop of endogenous miRNA precursors, artificial miRNAs (amiRNAs) are designed and processed in vivo to target the genes of interest. The strategy of expressing amiRNAs was first adopted to knock down endogenous genes for functional analysis. The technology is widely used in engineering antiviral plants and animals. Compared to conventional RNAi strategies, amiRNAs have many advantages: (1) Owing to the short sequence of amiRNAs, a long viral cDNA fragment is not required; thus, the full extent of off-target effects are avoided, and the biosafety of transgenic crops is increased compared to siRNAs from long hairpin RNA; (2) Tissue- or cell-specific knock out/downs of

genes of interest can be ~~realized~~ performed because of different tissue- or cell-specific promoters being used; (3) The relaxed demand on sequence length makes amiRNAs especially useful in targeting a class of conserved genes with high sequence similarities, like tandem arrayed genes, because a short conserved sequence is more easily found in these genes (Schwab., 2006).

Modified v~~V~~iruses ~~which~~ that haves ~~been modified and~~ used for gene silencing ~~the gene of interest~~ are~~is~~ summarized in Table 1. Tobacco mosaic virus (TMV) is one of the modified viruses ~~which~~ that ~~were~~ have been used for effective *pds* gene silencing in *Nicotiana benthamiana* plants. TMV was~~is~~ the first modified virus ~~for application of~~in which VIGS ~~methods~~ was applied to plants. The viral delivery leads to down~~-~~regulation of ~~transcript of~~the target gene through its homology~~-~~dependent degradation, so potential of VIGS for analysis of gene function was easily recognized. The tobacco rattle virus (TRV) ~~was~~ has been also modified to be a tool for gene silencing in plants. VIGS has been effectively applied in *N. benthamiana* and in tomato by using TRV vectors. The significant advantage of TRV-based VIGS in *Solanaceous* species is the ease of introduction of the VIGS vector into plants. The VIGS vector is placed between Rigth Border (RB) and Left Border (LB) sites of T-DNA and inserted into *Agrobacterium tumefaciens* (Liu et al., 2002; Ratcliff et al., 2001).

Another property of TRV is the more vigorous spreading all over the entire plant including meristem, and infection symptoms of TRV are mild. Modified TRV vectors such as pYL156 and pYL279 have a strong duplicated 35S promoter, and a ribozyme at C-terminus for more efficient and faster spreading. These vectors are also able to infect other plant species. TRV-based vectors haves been used by Liu et al. (2005) for gene silencing in tomato. Very recently, Pflieger et al. have shown that a viral vector derived from Turnip yellow mosaic virus [TYMV) has the ability to induce VIGS in *Arabidopsis thaliana*. VIGS of *N. benthamiana* using

**Comment [A3]:** Very recently when?? 2008? This reference is not included in the Reference list.

Potato virus X (PVX) was also achieved. 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. Geminivirus-derived vectors can be used for VIGS studies especially to study function of genes involved in meristem function. Tomato golden mosaic virus (TGMV) was used to silence a meristematic gene, proliferating cell nuclear antigen (PCNA) in *N. benthamiana*. The TGMV-based silencing vector had been used for also silencing of non--meristematic genes ~~silencing~~.

Satellite -virus-based vectors are also used for efficient gene silencing in plants only with the help of other helper viruses. This two-component system is called Satellite-virus-induced silencing system, SVISS (Fofana et al., 2004; Peele et al., 2001). Previously barley stripe mosaic virus (BSMV) was developed for efficient silencing of *pds* gene in barley. This system was then 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 replaced by DNA vector was used for plant gene cloning. β genome has been deleted for viral coat protein production defect. Each of the modified DNAs is used to synthesize RNAs by in vitro transcription. Recently, Brome mosaic virus strain has been modified for VIGS of *pds*, *actin,* and *rubisco activase*. These genes ~~were~~ have also been silenced in important model plants such as rice (Tao and Zhou, 2004). Steps for VIGS ~~have been~~are shown in figure 3:- p~~P~~rotocols for VIGS are as follows:

**Target sequence selection:**

siRNA Finder ~~si-Fi~~ (si-Fi~~siRNA Finder~~; http://labtools.ipk-gatersleben.de/) software could be used to select 250–400 nt sequence regions that are predicted to produce high numbers of silencing-effective siRNAs. When possible, select at least two preferably non-overlapping regions of the gene of interest for VIGS analysi~~e~~s. Observation of the same phenotype induced

by silencing using each of the two or more independent VIGS constructs is a good indication that the phenotype is due to specific silencing of the intended target gene, therefore allowing greater confidence in the obtained results. When attempting to silence an individual member of a gene family consider selecting the sequences from the 30 - or 50 -UTR regions, which are generally more variable than the CDS. This should minimize the risk of off-target silencing. On the other hand, in cases when a great deal of functional redundancy is expected among different gene family members, 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, at least one negative control 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.

**VIGS construct~~s~~ preparation:**

Clone the VIGS target sequences into the ~~for example~~ BSMV RNAc vector pCa-cbLIC (for example) via ligation independent cloning (LIC), in either sense or antisense orientation. Antisense constructs may be slightly more efficient in inducing gene silencing. Transform the sequence--verified 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 used: *Agrobacterium* cultures grown to a final OD600 of 1.2 and the cells will be pelleted by centrifugation and washed in ice-cold sterile 10% glycerol seven times in total. Electroporation can be done using the manufacturer's pre-set conditions for *Agrobacterium* i.e. one 2.2 kV pulse. Plate an aliquot of the transformation mixture on LB agar supplemented with 25 µg/ml gentamycin and 50 µg/ml kanamycin. As BSMV requires all three genomic segments, RNAa, RNAb and RNAc, for successful infection it is necessary to also

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produce *A. tumefaciens* GV3101 strains containing pCaBS-α (BSMV RNAα) and pCaBS-β (BSMV RNAβ).

## Preparation of virus inoculum and infecting target plants with engineered virus:

Prepared engineered virus introduced into the leaf of dicot plants (for example well studied *Nicotiana benthamiana*) via agroinfiltration. For *N. benthamiana* agroinfiltration, grow 5 ml cultures (LB supplemented with 25 µg/ml gentamycin and 50 µg/ml kanamycin) of *A. tumefaciens* strains carrying pCa-cbLIC VIGS constructs overnight at $28^0$C with constant shaking at 220 rpm. For each BSMV RNAc construct, BSMV RNAα and RNAβ constructs in 5 ml cultures will also be required. Pellet the *A. tumefaciens* cells at 2500 rcf for 20 min, re-suspend in infiltration buffer [10 mM MgCl2, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.6, and 150 µM acetosyringone] to a final optical density at 600 nm (OD600), and incubate at room temperature without shaking for 3 h or longer. Mix *A. tumefaciens* strains carrying BSMV RNAα, RNAβ, and RNAγ strains together in 1:1:1 ratio and pressure infiltrate the bacteria into the abaxial side of fully expanded leaves of approximately 25–30 days old *N. benthamiana* plants using a needleless 1-ml syringe. Use 0.5–1 ml of *Agrobacterium* suspension per leaf and aim to infiltrate the whole area of each leaf.

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## Assessment of virus-induced gene silencing:

Successful silencing of ~~the~~ target~~s~~ gene<u>s</u> in the VIGS construct-infected plants is assessed using quantitative reverse-transcription PCR (qRT-PCR). The primers used for this purpose should bind outside the region targeted for silencing.

## Viral infection to the plant and disease assessment:

After confirming the turning off of <u>the</u> target gene<u>,</u> ~~one has~~<u>it is necessary</u> to infect the host (plant) from the susceptible virus for the disease assessment. Early attempts to validate VIGS

technology used Tobacco mosaic virus (TMV) and Potato virus X (PVX). Genes were targeted ~~that~~ to produce~~d~~ distinctive phenotypes, such as silencing of GFP in transgenic tobacco expressing GFP (Figure 4), the photo-bleaching of leaves caused by a loss of carotenoid pigments when phytoene desaturase (pds) was disrupted (Kumagai et al., 1995; Ruiz et al., 1998). Other examples targeted the chlorophyll biosynthetic enzyme, resulting in plant chlorosis (Kjemtrup et al., 1998), and the cellulose synthase gene, resulting in a modification of plant cell walls (Burton et al., 2000). With the initial success of VIGS, researchers began targeting essential genes (Peele et al., 2001) such as those involved in plant resistance (Peele et al., 2001) encoding metabolic enzymes, increasing crop yield, or plant growth and development. For example, when a VIGS vector constructed with Tobacco rattle virus (TRV) was modified with the EDS1 gene required for N-mediated resistance to TMV (Peart et al., 2002), the inoculated plants had an enhanced susceptibility to TRV.

**Next generation VIGS with CRISPR/Cas system**

Virus-induced gene silencing has made a tremendous impact in plant biology by silencing and then identifying endogenous genes. However, with one of the most recent and promising genetic tools, the CRISPR/Cas DNA system, it is now possible for targeted genome editing and precise knocking out of entire genes. In recent studies, CRISPR/Cas9 was used to edit plant genomes such as rice, *N. benthamiana* and *Arabidopsis* for heritable changes (Nekrasov et al., 2013; Shan et al., 2013). The procedure is simple, requiring only transgenic plants expressing ~~cas9~~ Cas9 and guide RNA (gRNA, t~~). (T~~he technical terms are explained below). Additionally, the genetic modifications are present in subsequent generations. The VIGS system, besides its ability to

silence genes, has found an important application in the CRISPR/Cas editing system. It can be used as a vehicle to transport the CRISPR/Cas editing system into plants ~~system~~.

It is expected that CRISPR/Cas will transform the way plant traits are modified in the future. Although this technology is new, a number of proof of concept studies in model plants have shown the ~~its~~ potential as a powerful gene editing technology. The efficiency, accuracy and flexibility of the CRISPR/Cas9 genome engineering system has been demonstrated in various eukaryotes such as yeast, zebrafish, and worms (DiCarlo et al., 2013; Friedland et al., 2013; Hwang et al., 2013; Mali et al., 2013). The potential applications have been growing rapidly and include the cutting-edge application of gene editing in the germlines of humans and other organisms (Mali et al., 2013). This method was recently adopted in plant systems in various transient experiments or in transgenic plants, and is becoming the method of choice for plant scientists.

Like RNA interference, the CRISPR/Cas gene-editing technology was derived from a naturally occurring plant-defense mechanism. It provides a form of acquired immunity to the cleavage of DNA present in certain prokaryotes and confers resistance against foreign genetic elements such as phages and plasmids. It is based on the type II CRISPR (clustered regulatory interspaced short palindromic repeats) (Figure 4). CRISPR is a sequence of short, ~~repetitious~~ repetitive segments followed by a short segment of spacer DNA. The spacer DNA could be from previous exposures to a virus, plasmids, or bacteria ~~um~~. Evidence that the source of the spacers was a bacterial genome was the first hint of the CRISPR's role in an adaptive immunity analogous to RNA interference. It was soon proposed that the spacers identified in bacterial genomes served as templates for RNA molecules that the bacteria transcribed immediately after an exposure to an invading phage. Further studies revealed that an important protein called Cas9

was involved, together with the transcribed RNA, to recognize the invading phage and cut the RNA into small pieces (crRNA) in the CRISPR system (Horvath and Barrangou, 2010; Jiang et al., 2013; Ran et al., 2013). CRISPRs are found in almost 90% of the sequenced Archaea and up to 40% of bacterial genomes (Horvath and Barrangou, 2010). Native bacterial CRISPR RNAs also can be altered into a single gene known as a single-guide RNA (sgRNA) (Jinek et al., 2012; Schaeffer and Nakata, 2015). Using sgRNA has made the system more flexible, allowing it to simplify genome editing by combining sgRNA and Cas-9 in a heterologous system. Applying the CRISPR/Cas9 system in plants uses both components; the Cas9 enzyme catalyzes DNA cleavage and the sgRNA recruits Cas9 to the target site. This site is usually located about 20 nucleotides before the protospacer motif and cleaves the DNA. The natural mechanism plants use to reattach the cleaved ends of DNA is called non-homologous end joining (Xie et al., 2014) and usually results in a mutation either by frameshift, insertion/deletion, or insertion of a stop codon. Therefore, by simply designing an sgRNA with a complementary sequence, virtually any gene can be edited with this heterologous system.

**Integration of VIGS and CRISPR/Cas9**

As mentioned in the previous section, recognition of the usefulness of the TRV-based VIGS vector in functional genomics was followed by its use to deliver the components for genome editing into plants. TRV is ideally suited since it can systemically infect a wide range of important crop plants. Moreover, TRV is widely used to transiently infect any plants using the TRV-VIGS system, so the protocols are well established. The ability of TRV to infect the plant meristems makes it an ideal candidate for delivery of CRISPR/Cas9 since any seeds derived from these plants will have the induced modifications that are heritable. This bypasses the need for time-consuming transformations or tissue culture to obtain mutant seeds.

In a recent study, TRV--delivered sgRNA molecules were used to edit the *phytoene desaturase* (PDS) gene in *N. benthamiana* (Ali et al., 2015). To develop the system, researchers used *Agrobacterium*-mediated transformation protocol to generate transgenic lines of *N. benthamiana* that overexpressed Cas9. Next, they modified the RNA2 genome of TRV for sgRNA delivery. The sgRNA directed to target the PDS was expressed by a promoter derived from *Pea early browning virus* (PEBV). Subsequently, they reconstituted the functional TRV virus by introducing RNA1 of its bipartite genome into tobacco leaves by agro-infiltration. After two weeks, they assayed the plants and found the genomic modifications in systemically infected leaves. Importantly, the genetic modification for the PDS gene was present in the progeny due to infection of the meristematic cells and subsequent seed transmission. The demonstration of TRV for virus-mediated genome editing suggests the possibility of modifying a wide variety of plant species by using other RNA viruses as vectors. Recently, the use of CRISPR/Cas9 was extended to include a DNA virus, *Cabbage leaf curl virus* (CaLCuV) in the genus *Geminivirus*. Since DNA viruses replicate in the nuclei of plant cells, expression of sgRNA should be more efficient since genome editing occurs in the nucleus (Yin et al., 2015). Moreover, CaLCuV has a number of hosts in the Brassicaceae including cabbage, cauliflower and *Arabidopsis*. It also infects *N. benthamiana* and other solanaceous crops.

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## CONCLUSION

The discovery of RNA interference (RNAi), the process of sequence-specific gene silencing initiated by double stranded RNA (dsRNA), has broadened our understanding of gene regulation and has revolutionized methods for genetic analysis. Gene expression is regulated by transcriptional and post-transcriptional pathways, which are crucial for optimizing gene output

and for coordinating cellular programs. In plants, 20-24 nltd RNAi regulate gene expression networks necessary for proper development, cell viability and stress responses. Gene silencing techniques represent great opportunities for plant breeding. Several practical applications in economically important crops are possible as well as research on gene function and expression. RNAi stability in plants is a very important feature to be accessed in the near future as well as the development of tissue--specific and inducible promoters. These are two crucial points for the establishment of this technology as a marketable option. Control of metabolic pathways will also represent a major challenge when trying to obtain plants with altered levels of specific metabolites. The use of artificial miRNAs to engineer viral resistant plants also shows great potential. Continuing research on GS in woody plants will probably include plant protection to multiple pathogens (viruses, bacteria), silencing of specific metabolic pathways (lignin synthesis, ethylene, allergens, caffeine and others), improvement of fruit and wood quality, production of secondary metabolites, and developmental and reproductive trait alteration in plants (induced male sterility and self-compatibility). The ability to switch off genes and interfere with expression patterns in plants, provided by gene silencing techniques, will probably represent a great impact in woody plant breeding.

**Comment [A4]:** Please indicate what does nltd stand for



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847

848 **Table1:** Plant viruses used as VIGS vectors, the nature of their genomes and their important hosts

**Comment [A5]:** Most of these references are not listed in the References section.

| 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* | Fofana et al., *2004* |
| **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* | Igarashi et al., 2009 |
| **Barley stripe mosaic virus** RNA virus, tripartite | Hordeivirus | Barley, wheat, oat, maize, spinach | *Hordeum vulgare,, Triticum aestivum* | *Pds, TaEra1* | Holzberg et al., 2002; Manmathan et al., 2013 |
| **Bean pod mottle virus** RNA virus, bipartite | Cucumovirus | Phaseolus vulgaris, Glycine max | *G. max* | *Pds, GmRPA3* | Atwood et al., 2014; Zhang and Ghabrial, 2006 |
| **Brome mosaic virus** RNA virus, tripartite | Bromovirus | Barley | *Hordeum vulgare, Oryza sativa and Zea mays* | *pds, actin 1, rubisco activase* | Ding et al., 2006 |
| **Cabbage leaf curl virus** DNA virus, bipartite | Begomovirus | Cabbage, broccoli, cauliflower | *A. thaliana* | *gfp, CH42, pds* | Turnage et al., 2002 |
| **Cucumber mosaic virus** RNA virus, tripartite | Cucumovirus | Cucurbits, S. lycopersicon, Spinacia oleracea | *G. max* | *chs, sf30h1* | Nagamatsu et al., 2007 |
| **Pea early browning virus,** RNA virus, Bipartite | Tobravirus | Pisum sativum, Phaseolus vulgaris | *P. sativum* | *pds, uni, kor* | Constantin et al., 2004 |
| **Poplar mosaic virus** RNA virus, monopartite | Carlavirus | Poplar | *N. benthamiana* | *gfp* | Naylor et al., 2005 |
| **Potato virus X** RNA virus, monopartite | Potexvirus | Solanum tuberosum, Brassica campestris ssp. rapa | *N. benthamiana, A. thaliana* | *gus, pds, DWARF, SSU, NFL, LFY* | Ruiz et al., 1998 |
| **Satellite tobacco mosaic virus** | RNA satellite virus | Nicotiana glauca | *N. tabacum* | *Several genes* | Gosselé et al., 2002 |

| | | | | | |
|---|---|---|---|---|---|
| RNA virus, satellite | | | | | |
| **Tomato bushy stunt virus,** RNA virus | Tombusvirus | *S. lycopersicon, N.benthamiana* | *N. benthamiana* | *gfp* | Hou and Qiu, 2003 |
| **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* | Huang et al., 2009 |
| **Tobacco mosaic virus** RNA virus, monopartite | Tobamovirus | *N. tabacum* | *N. benthamiana, N. tabacum* | *pds, psy* | Kumagai et al., 1995 |
| **Tobacco rattle virus** RNA virus, bipartite | Tobravirus | Wide host range | *N. benthamiana, A. thaliana, S. lycopersicon* | *pds, rbcS, FLO/LFY (NFL) Sllea4* | Liu et al., 2002b; Ratcliff et al., 2001; Senthil-Kumar and Udayakumar, 2006 |
| **Tomato golden mosaic virus,** DNA virus, bipartite | Begomovirus | *S. lycopersicon* | *N. benthamiana* | *su, luc* | Peele et al., 2001 |
| **Tomato yellow leaf curl China, virus–associated b** DNA satellite | Begomovirus | *S. lycopersicon* | *N. benthamiana S. lycopersicon, N. glutinosa, N. tabacum* | *pcna, pds, su, gfp* | Tao and Zhou, 2004 |
| **Turnip yellow mosaic virus,** RNA virus, monopartite | Tymovirus | Brassicaceae | *A. thaliana* | *pds, lfy* | Pflieger et al., 2008 |

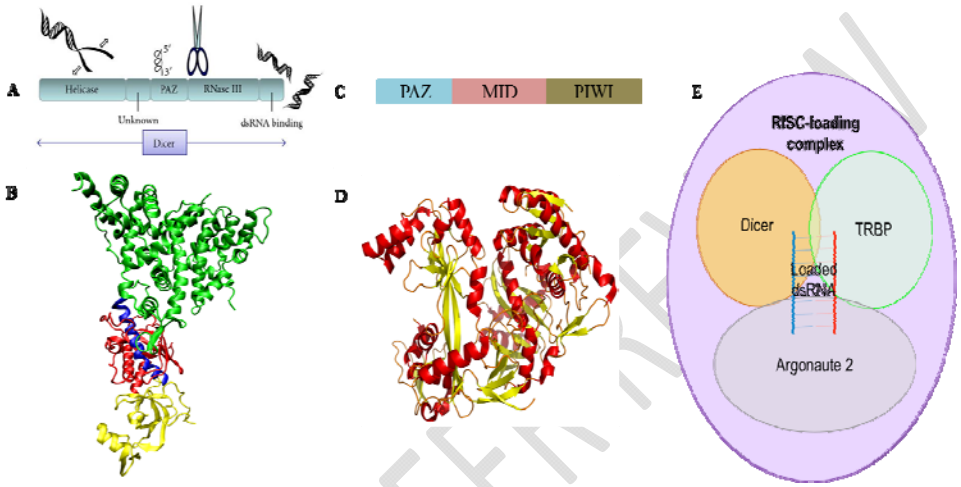



**Figure 1.** Principal components of RNA interference. (A) Schematic representation of all predicted domain organization on the polypeptide chain of Dicer protein. Helicase: N-terminal and C-terminal helicase domains. PAZ: Pinwheel-Argonaute-Zwille domain. RNase III: bidentate ribonuclease III domains. (B) Tertiary structure of the Dicer protein from the source *Giardia intestinalis*. The RNase III, PAZ, platform and connection helix are shown in green, yellow, red and blue respectively (Adapted from Macrae et al., 2006). (C) Schematic representation of all predicted domain organization on the polypeptide chain of Argonaute protein. (D) Tertiary structure of the Argonaute protein from the source *Pyrococcus furiosus* (PDB 1UO4). (E) Hypothetical complete RISC-loading complex, allows loading of dsRNA fragment generated by Dicer to Argonaute protein by the assistance of TRBP.

**Comment [A6]:** Do the authors have permission to use this figure? Even if the figure has been modified, permission is required.

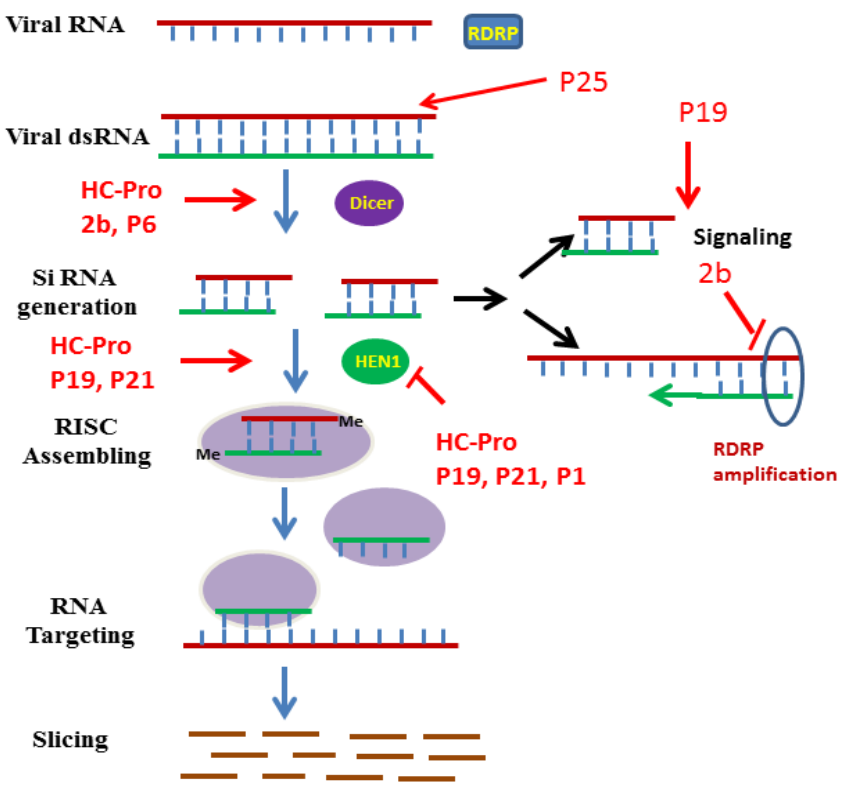
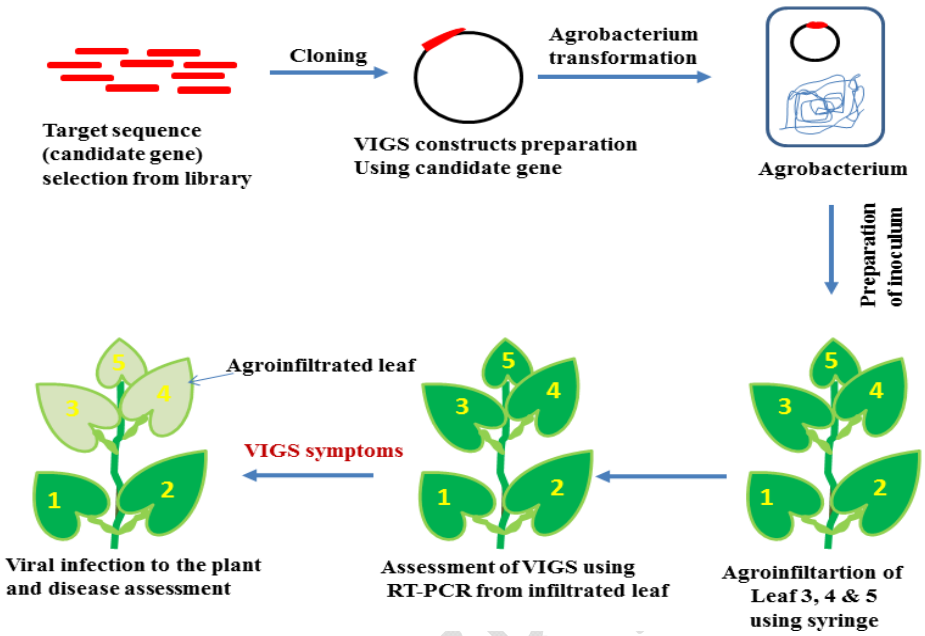



**Figure 2.** Viral RNA silencing in plants and its counter defense.

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900 **Figure 3.** Steps of virus-induced gene silencing (VIGS). VIGS starts by ~~the~~ cloning ~~of~~ the target gene
901 fragment (200-1300 bp) into a virus infectious cDNA, which is in a binary vector under the control of the
902 CaMV 35S promoter. The recombinant virus construct is then transformed into ~~agrobacterium~~
903 *Agrobacterium* (*Agrobacterium tumefaciens*) for ~~*agrobacterium*~~ *Agrobacterium*-mediated virus infection.
904 VIGS will target ~~to~~ the virus carrying the~~ied~~ host gene fragment as to the viral genome, and also the
905 endogenous host gene target.
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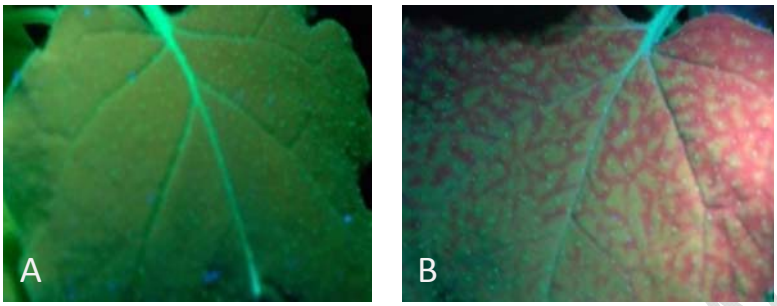


**Figure 4. Virus-induced silencing in 16C trasgenic *N. benthamiana* for GFP.** Leaves examined under a long-wavelength UV light at 7 weeks post-inoculation. (A) Non~~Un~~-inoculated leaves showing GFP fluorescence. (B) Leaves co-infiltrated with 35S-sGFP and a pBIC-35S-empty vector induced silencing. The non-inoculated upper leaves showing development of red trails due to systemic silencing of GFP.