Application of Virus-derived small interfering RNAs (vsiRNAs) in rice viruses with insect vectors, especially *Rice grassy stunt virus*

MUHAMMAD ARIF¹, SAIF UL ISLAM¹, MUHAMMAD ADNAN², MUHAMMAD ANWAR³, ZUJIAN WU¹*

¹ State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Fujian Province Key Laboratory of Plant Virology, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China.

²Key Laboratory of Biopesticide and Chemical Biology of Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China.

³College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China.

*Corresponding author: ZUJIAN WU: Email: 2141904001@m.fafu.edu163.com

Abstract:

*Rice grassy stunt virus* (RGSV) a member of *Tenuivirus* family, is very potent and destructive which effects rice crop in many countries, particularly China. Non coding RNAs have important functions in development and epigenetic regulation of gene expression in numerous organisms. There is three type of small non coding RNAs have been found in eukaryotes, which are small interferring RNAs (siRNAs), microRNAs (miRNAs) and piwi interacting RNAs (piRNAs). Small RNAs (sRNAs) origination is from the infecting virus which is known as virus-derived small interfering RNAs (vsiRNAs), has responsibility for RNA silencing in plants. Virus-induce d gene silencing (VIGS) is mainly dependent on RNA silencing (RNAi). Interestingly, RNA silencing happens in plants during viral infections. RNAi technique showed significant results in *Nephotettix cincticeps*. RNAi technique demonstrated the gene silencing of planthopper *Nilaparvata lugens*. The proteins P5, pcf4, Dnj, psn5, and pn6 act as potential movement proteins and serve as silencing suppressors for RGSV. VsiRNAs originate from dsRNA molecules which require Dicer-like (DCL) proteins, RNA dependent RNA polymerase (RdRP) proteins, and Argonaute
(AGO) proteins. RdRP uses ssRNA for perfect RNA amplification process and can also be used for DCL dependent secondary vsiRNA formation. VSRs interfere with the movement of signals during silencing mechanism. Moreover, intercellular movement of viruses is facilitated by virus-encoded movement protein. RNAi is found in many eukaryotes which are related to transcriptional or post-transcriptional regulation by gene suppression. Transcription is bidirectional in ssDNA viruses which are originated from dsRNA molecules. In this review, we highlighted the biology of Rice grassy stunt virus and its insect vector and its silencing suppressors. This work will be helpful for plant virologists to understand the whole biogenesis mechanism for rice viruses especially RGSV.

**Key Words:** RGSV; movement proteins; gene silencing; transgenic mechanism; virus-induced small interfering RNA; RNA silencing pathway; antiviral plant defenses

### 1. Introduction

*Rice grassy stunt virus* (RGSV) is a member of the genus *Tenuivirus* having six other types as well. RGSV is a devastating plant pathogen whose transmission occurs by brown insect planthopper *Nilaparvata lugens*, *Nilaparvata* species, *N. bakeri* and *N. muiri* [87]. Recently more than 485,000 hectares of rice field severely infected by RGSV or with a combination of RSV in Vietnam lead to loss of 828,000 tons of rice [87]. The symptoms of this viral infection are yellowing of leaves, stunted plant growth and increase the number of tillers, leave narrowing, along with mottling symptoms on newly emerged rice leaves [77, 80].

*Rice grassy stunt virus* (RGSV) and *Rice stunt virus* (RSV) have been found to coinfect rice with the same pattern of *Rice ragged stunt virus* (RRSV). Both viruses during co infection created 21 nt sizes capped RNA from RRSV. 245 RRSV-RGSV and 102 RRSV-RSV chimeric mRNA were cloned and sequenced. During analysis of these sequences, a theory was consistent with old data about these viruses, from which capped front RNAs possessing a 3’ end complimentary with the viral template are chosen and upon base pairing prime possessive transcription immediately or from one cycle to several cycles of priming and realignment. It was quite interesting that RSV found to have a high ability for using the repetitive prime and realign rather than RGSV, even same front RNA derived from same RRSV RNA [50].

MicroRNAs (miRNAs) are essential modulators for plant development and host virus interactions. There is a small number of theories have been reported in which specific miRNAs
have involvement during viral infection or host susceptibility. [122] found that RRSV infection increases the miR319 accumulation and obstructs the expression of miR319 regulated TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP21) genes in the host. Transgenic rice plants which are overexpressing miR319 or down regulating TCP21 showed disease like phenotypes and exhibited the higher susceptibility to RRSV with wild type plants. In comparison, mild diseases symptoms were detected in RRSV infected lines with overexpressing TCP21. There was a decrease in endogenous Jasmonic acid level in RRSV infection and overexpression of miR319 along with down regulated expression of JA biosynthesis signal related gene in rice. Treatment of rice with methyl jasmonate (MeJA) promoted the disease symptoms caused by RRSV and also showed a reduction of virus accumulation. Induction of miR319 through RRSV infection in rice suppressors JA mediated defense for facilitation of virus symptoms and symptoms development.

*Rice stripe virus* (RSV) belongs to Tenuivirus genus, and still, this genus has not been classified into any family yet [30]. RGSV is also a member of this genus which causes disease propagation by the circulative and persistent way with the help of small brown planthopper (Laodelphax striatellus). Insect vectors are responsible for replication of plant viruses and long distance movement of viruses by the viruliferous insects and pests. *Rice gall dwarf virus* (RGDV) is also very important virus of rice crop which attacks transovarially by insect vectors to a new generation with the help of eggs.

RGSV is recently the most significant and destructive virus of rice family which causes plant growth stunting and profuse tillering which greatly reduce the yield of the rice crop. Many varieties and new lines have been grown in the form of resistance to control *N. lugens* in many Asian countries, but every time one new insect vector biotype has been observed after planting the resistant variety or cultivar [22]. RGSV has 6 RNA segments, and 12 open reading frames (ORFs). RNAs 1, 2, 5 and 6 of RGSV quickly correspond to RNAs 1, 2, 3 and 4 in *rice stripe virus* (RSV) which is a member of *Tenuivirus* [69].

Three RGSV encoded protein functions are known. RNA1 is encoded by pC1 (339.1-kDa) by the complementary sense, and it acts as RNA dependent RNA polymerase (RdRp), which has similarity to RdRp of RSV with 37 ± 9 % amino acid sequence identity which is over 2,140 amino acid residues. The Protein pC5 having size 35.9 kDa, is mainly encoded by vcRNA5 that is nucleocapsid protein which accumulates in RGSVi and viruliferous brown planthoppers [13]. The protein pC6 (36.4 kDa) which is proposed to act as a movement protein, is mainly encoded
by vcRNA6 and it can trans-complement for the detection of TMV cell-to-cell extended movement [35]. It is supposed that the RGSV protein functions are going to be deduced by RSV counterparts, but its functional authentication is very important which is given by the sequence of the low amount of amino acid characterization between these two proteins [121]. Many viruses are obligate infectious agents, and their life cycle, viral protein expression, viral genome replication and virion assembly are responsible for the host cell functions. Plant viruses can change the characteristics of endogenous sRNAs which may lead to the formation of other sRNAs that are different from their original genomes (viral sRNAs; vsiRNAs). Similarly, it was confirmed that it helps to activate the RNA based gene silencing responses in plants. Several times, it has been proved that titre reduction in invading virus leads to regaining of non-inoculated leaves [88] [91].

1.1 Planthoppers

Planthoppers belong to kingdom Animalia, phylum Arthropoda, class Insecta, order Hemiptera and family Delphacidae which has 2,000 species worldwide. Planthoppers are important vectors which transmit 3% of all plant viruses. Nucleorhabdovirus, Tenuivirus, Phytovirus, and Oryzavirus are virus families in which plant hoppers act as vectors to transmit the viruses in plants [36]. Nilaparvata lugens brown plant hopper acts as an important plant virus vector in Southeast Asia. RGSV and rice ragged stunt virus (RRSV) cause economically important diseases in which planthoppers transmit the diseases via persistent manners [73]. Silencing of planthopper N. lugens genes was demonstrated via RNAi technique, which was done by different methods like injection (calreticulin, cathepsin-B, beta2) [48], ingestion of V-ATPase-E, 21E01 [44] and artificial feedings (trehalose phosphate synthase, NITPS) [10]. RNAi has become a dominant technique to evaluate the gene function in insects nowadays. [48], they performed RNAi strategy through microinjection of dsRNA in brown planthopper (N. lugens). They selected three genes with dissimilar expression patterns to check the RNA interference mechanism. Universally expressed Calreticulin gene and cathepsin-B, beta2 showed 40% reduction of gene expression after injection at 4th day but 25% reduction of Nlβ2 (central nervous system-specific gene) at 5th day. (Lu et al, 2015) [55] stated in their experiment that NNNgR gene showed specific expression level in planthopper ovary and level of mRNA increased subsequently adult female emergence. Protein NNNgR showed ovary-specific expression mechanism during western blot analysis and
also showed consistency with N/VgR transcript detection. Injection with N/VgR (dsRNA) considerably disturbed the N/VgR gene, that directed to decrease of NVg protein level in insect ovary.

Silencing of ecdysone receptor was done in species of planthoppers, *N. lugens, L. striatellus*, the vector for *Rice stripe virus* (RSV) and *Sogatella furcifera*, the vector of *Southern rice black-streaked dwarf virus* (SRBSDV) which showed phenotypic defects and mortality of molting nymphs as shown in (Table 1) [107]. Silencing of coronatine insensitive 1 (COI1) in rice plants not only improved the defense responses in plants but also enlarged the *N. lugens* susceptibility of feeding the rice plants [113]. Silencing of chitin synthase (CHS1, CHS1a) showed results as elongated wing pads, crimped cuticle phenotypes and also insect lethality, in which phenotypes were triggered by injection of CHS [101]. [112] reported that reporter genes which are involved in siRNA and miRNA pathways in brown planthoppers, the miRNA pathway showed brown plant hopper metamorphosis as weakening of Argonaute (AGO1) and Dicer (DCR-1) genes extensively and also showed a reduction of ecdysis. DCR-2 gene disclosed 55% minimization of gene expression in *N. lugens* after feeding, and also no changes were seen insect development [126].
Figure 1: Application of RNAi against viruses. Feeding on transgenic plants that carry RNAi constructs to confer resistance to the insects.
Figure 2: Application of RNAi against viruses. Viral small RNA expression motivates antiviral silencing in plants.

Glutamate synthase (GS) knockdown by RNAi also decreased the fecundity of *N. lugens* by almost 64.6%, disrupted ovary development and showed vitellogenin (Vg) inhibition [117]. DCR plays an important role in *N. lugens* for the regulation of oogenesis in the telotrophic ovary [125]. Arginine kinase and Hsp70 plays a critical role in survival, and triazophos increases the thermotolerance in brown planthopper [33]. Two species of *N. lugens* were targeted by RNAi, GSTe1, and GSTm2 that significantly increased the fourth-star nymph’s sensitivity to pesticide chlorpyriphos [130]. Enolase gene (Eno1) silencing revealed down-regulation of mRNA level
significantly in *N. lugens* and also decreased the egg laying capacity and population size during next generation of the planthopper [100]. Silencing of N-acetylhexosaminidase gene causes the molting and death of brown planthopper [108]. Chitinase silencing showed significant results in small planthopper (*L. striatellus*) of maximum mortality and Halloween gene Shade (ShD) knockdown minimized the expression of EcR gene and affected the nymphal lethality [54] [40].

**Table 1.** Reported cases of gene silencing in insect vector plant hoppers for plant pathogens.

<table>
<thead>
<tr>
<th>Planthopper</th>
<th>Targeted Genes</th>
<th>Delivery method</th>
<th>Phenotype after silencing</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nilaparvata lugens</em></td>
<td><strong>Ecysone receptor (EcR)</strong></td>
<td>Artificial feedings</td>
<td>Reduced survival rate of the offspring</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td><em>Vg receptor</em></td>
<td>Injection</td>
<td>Fecundity</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td><em>Enolase gene (Eno1)</em></td>
<td>Injection</td>
<td>Decreased egg laying</td>
<td>[100]</td>
</tr>
<tr>
<td></td>
<td><em>Bicaudal-C</em></td>
<td>Injection</td>
<td>Role in oogenesis and oocyte maturation</td>
<td>[119]</td>
</tr>
<tr>
<td></td>
<td><em>Acyl-coenzyme A oxidase (ACO)</em></td>
<td>Artificial feedings</td>
<td>Decreased the reproduction and population growth</td>
<td>[52] [127]</td>
</tr>
<tr>
<td></td>
<td><em>Glutamine synthetase (GS)</em></td>
<td>Injection/Artificial feedings</td>
<td>Decreased the reproduction and population growth</td>
<td>[118]</td>
</tr>
<tr>
<td><strong>Population Growth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NIHsp90</strong></td>
<td>Injection</td>
<td>Deduced survival, role in thermotolerance</td>
<td>[57]</td>
<td></td>
</tr>
<tr>
<td><strong>N-acetylhexosaminidase</strong></td>
<td>Injection</td>
<td>Failure of the nymphs to molt which eventually led to death</td>
<td>[108]</td>
<td></td>
</tr>
<tr>
<td><strong>Trehalose-6-phosphate synthases (TPS1 and TPS2)</strong></td>
<td>Injection</td>
<td>Chitin metabolism and increased molting deformities and mortality rates/regulates Vitellogenin synthesis and egg development</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td><strong>P450 proteins (CYP6AY1 and CYP6ER1)</strong></td>
<td>Injection</td>
<td>Imidacloprid resistance</td>
<td>[2]</td>
<td></td>
</tr>
<tr>
<td><strong>L. striatellus Cytochrome P450 monooxygenase CYP353D1 v2</strong></td>
<td>Artificial feedings</td>
<td>Sensitivity of <em>L. striatellus</em> to imidacloprid</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td><strong>Cuticular protein (CPR1)</strong></td>
<td>Injection</td>
<td>Reducing the ability to transmit <em>rice stripe virus</em> (RSV)</td>
<td>[49]</td>
<td></td>
</tr>
<tr>
<td><strong>(Leaf Laccase-2)</strong></td>
<td>Injection</td>
<td>High mortality</td>
<td>[65]</td>
<td></td>
</tr>
</tbody>
</table>
**1.2 Leafhoppers**

Leafhoppers belong to Cicadellidae and Delphacidae family, which also act as vectors to transmit plant viruses and phytoplasma in many crops by persistent, semi-persistent and propagative manners. *Bunyaviridae, Geminiviridae, Reoviridae, and Rhabdoviridae* are the virus families for which leaf hopper serve as vectors [36]. *Nephotettix* species is a green planthopper which is also known as green paddy leaf hopper which has become an important rice pest in Asia and caused the *Tugro rice virus* disease.

RNAi technique showed good results in *Nephotettix cincticeps*. Injection of dsRNA of laccase-2 into the first instar of nymphs of leafhopper leads to maximum death rates and also body depigmentation [65]. Peptidoglycan recognition protein (PGRP-LC) knockdown in black leafhopper *Graminella nigrifrons* showed 90% or more death rates [12]. Reducing the expression of Hexamine in beetle leafhopper *Circulifer tenellus* which acts as a vector to transmit cutoviruses, showed the reduction of phenol oxidase-like activity and resulted in maximum mortality [27].

Here we have mentioned some roles of viral suppressors for *Rice grassy stunt virus*.

**2.1.1 Role of p5 as a viral suppressor**

A cDNA library was screened with the help of GAL4 based yeast two hybrid system by applying RGSV p5 as bait [110]. One important host factor which interacts with RGSV p5 was found to be CBL interacting protein kinase25 (OsCIPK25), which participate in plant specific CBL-CPK Ca$^{2+}$ signaling network. This interaction of RGSV p5 with OsCIPK25 and also OsCIPK5 that have a close relation with OsCIPK25 was established by its bimolecular fluorescence complementation assay and cellular co-localization in *N. benthamiana* cells. Importance of CIPKs during the ion homeostasis regulation and also RGSV symptoms similarity with deficiency of Potassium in rice field showed the potassium content of RGSV infected rice and concluded that content much low as compared to healthy rice.

In this study, authors confirmed that RGSV p3 has no impact on a VSR and no VSR activity of p5 was found over RGSV p3 and not even in p5 localized pattern from Tobacco *Nicotiana*.
*benthamiana* plant cells. In a p5-p5 viral sense, p5 interacts with itself and encodes RNA3 with p3. P5-p3 interactions were found by bimolecular fluorescence complementation (BiFC) assay, co-immunoprecipitation (Co-IP) assays and subcellular co-localization that helps to confirm the p5-p3 interaction. P5-p3 interaction needs N-terminal (amino acid residues 1 to 99) and C-terminal (amino acid residues 94 to 191) domains of p5 by using Yeast 2 Hybrid system. Interestingly, when p5 and p3 were expressed together, then PVX accumulation and pathogenicity was found [120].

### 2.1.2 Role of Pc4 as putative movement protein

The use of Pc4 as bait (which is RSV putative movement protein) by the Gal4-based yeast-two-hybrid system for screening the cDNA of rice, produced a maximum number of positive colonies. Ten independent proteins also responded by sequence analysis. Amongst the ten proteins, J protein and small Hsp protein were used to identify the interaction. By communoprecipitation, it was confirmed that pc4 has interaction with these two proteins. RSV has 4 single standard genomic RNA which is called as RNA1, 2, 3 and 4 with their size in reducing style. RNA1 has one big ORF which encodes a protein of 337 kDa molecular weight that was thought to be (RDRp) which is linked with RSV filamentous ribonucleoprotein. RNA2 and 4 have ORFs at the 5′ half of complementary viral sense and are ambisense (or antisense). Pc2 and P2 are non-structural proteins which are encoded by RNA2, p3 is another non-structural protein encoded by RNA3, while RNA4 encodes pc4 which is disease-specific (SP) [58].

Plant viruses during their infection cycle involve a period of movement from already infected cell to neighboring or adjacent cells via Plasmodesmata (PD). Some virus-encoded proteins called movement proteins (MPs) help in the cell to cell movement. Sometimes PD is replaced by tubules formed by MPs to aid the passage of virions [58]. The MPs are only reversibly and transiently dilate the openings of PD to facilitate the transport of ribonucleic acids or viral nucleic acids-protein complexes [97]. Also, the MPs have certain features in common with a set of endogenous host factors called non-cell autonomous proteins (NCAPs) regarding movement from cell to cell [82]. Hence it is openly believed that MPs of viruses utilize the pre-existing pathways to fulfill their function.

Moreover, expression of a non-cell-autonomous pathway protein (NCAPP), a dominant-negative mutant form of NtNCAPP1, eliminated the cell to cell transport of MP of TMV and specific
NCAPs like CmPP16 [42]. Consequently, it is clear that MPs of viruses require a series of interactions with the host factors for various viral functions [60].

2.1.3 Role of Pns12 as silencing suppressor
Many Plants have a strong defensive mechanism regarding RNA silencing against invasive nucleic acids of many viruses, transgenes, and transposons. RNA silencing suppressors have been used in many plants viruses as a counter defense to obstruct one or several points of silencing pathway. RGDV (Rice Gall Dwarf Virus) of Pns12 has been shown as a suppressor in transgenic *Nicotiana benthamiana* line 16c on green fluorescent protein. It was confirmed that RGDV of Pns12 had no interaction to that induced by dsRNA but could act as a suppressor of local silencing with sense RNA. Similarly, *Potato virus X* pathogenicity in *N. benthamiana* can be enhanced by the expression of Pns12. It was found that Pns12 can be used as a viral suppressor of RNA silencing that can lead to the formation of dsRNA in RNA silencing pathway. Also, Pns12 is localized in the nucleus of *N. benthamiana* leaf cells [104].

2.1.4 Pns6 Role as putative movement protein
[104] The study of Wu et al., (2010) confirmed another protein Pns6 which is also putative movement protein in Rice *Ragged Stunt Virus* (RRSV), which shows silencing suppressor activity in co-infiltration assays by GFP in transgenic *Nicotiana benthamiana* line 16c. RRSV of Pns6 also showed local suppression by sense RNA, but no activity was found induced by dsRNA. It was the pioneer silencing suppressor found in rice. [104] took these two proteins together; Pns6 consists of 592 amino acids and has 65 kDa molecular weight which acts as RRSV silencing suppressor.

*Reoviruses* which infect plants are classified into three genera as *Fijivirus*, *Phytovirus*, and *Oryzavirus* [30]. RRSV is a member of genus Oryzavirus. The viral transmission is by persistent manner, by the vector brown planthopper. The plant family which is the main target of this virus is Gramineae. This viral disease was first discovered from 1976 to 1977 in Philippines and Indonesia. Afterwards, the disease spread to a large number of rice growing areas in far eastern and southeastern Asia and hampered rice production with severe yield losses. The virion of RRSV is an icosahedral particle, having a polyhedral core with flat spikes about 10 nm high and 20 nm wide surrounding. The genome of RRSV consists of 10 dsRNAs with a range of 1.2 to 3.9 kb molecular weights. The complete nucleotide sequence of every genomic segment has been determined which is denoted as S1-S10 [103].
2.1.5 Role of Pns9 as Silencing Suppressor

[4, 95] found that only one important process regarding RNA silencing or RNA interference RNAi which is found in many eukaryotic organisms, and can be found at transcriptional and post-transcriptional levels in the shape of gene suppression. SiRNA is integrated into the induced RNA silencing complex which is responsible for leading the translational repression or translational repression degradation by the specific sequence way as shown in Figure (1).

[67] confirmed that when hairpin forms then the expression of dsRNA becomes so simple that may lead to silence the pathogenic genes by many species which are invaded and also endogenous genes by expression. Hairpin RNA is derived from the gene for Pns9, a viroplasm matrix protein of *Rice gall dwarf virus* confers strong resistance to virus infection in transgenic rice plants.

2.1.6 Role of RDRs, AGOs and DCL enzymes as Silencing Suppressor:

MicroRNAs have become an important element for plant pathogenic interactions. Modulating miRNA function has been developed as a novel mechanism for production of virus resistance traits, but the miRNAs involvement in antiviral defense and its fundamental mechanism still remain indefinable. [106] previously confirmed that sequestration by Argonaute (AGO) proteins have a key role in regulating miRNA function during the antiviral defense pathways. The current study of these authors revealed the cleavage defective AGO18 complexes sequester microRNA528 upon viral infection. MiR528 negatively regulates the viral resistance in rice host via cleaving L-ascorbate (AO) oxidase messenger RNA, so reducing the AO mediated accumulation of reactive oxygen species. After the completion of viral infection, miR528 becomes specially associated with AGO18, which leads to elevated AO activity and also a higher basal accumulation of reactive oxygen species and boosted the antiviral defense. Authors develop a mechanism in which antiviral defense is enhanced by suppression of miRNA which regulates virus resistance negatively. This novel technique can be used for developing the engineer viral resistance plants.

RNAi has become a key antiviral defense mechanism in animals and Plants. RNA dependent RNA polymerase is the main part of RNAi which contribute importantly to resistance against plant viruses. The genome of species Arabidopsis is encoded by 4 DCL enzymes, the genetic study confirmed that DCL4, DCL2, and DCL3 enzymes hierarchical access by vsiRNA size class biogenesis \[[24] [78]\]. 21nt vsiRNAs are produced by a DCL4 enzyme which is most
important and basic sensor of viral dsRNA in which infected tissues have abundant size. In the absence of DCL4 and DCL2 enzyme which produces 24nt vsiRNAs DCL shows its efficacy to target the viral dsRNA and DCL4; Moreover DCL4 plays its role as a substitute to produce 22nt vsiRNAs. RNA silencing is originated from dsRNA which is processed into vsiRNAs through DCL2/3/4 in rice (Oryza sativa) as shown in Figure (3).

Generation of double stranded break (DSB induced small RNAs (diRNAs) are carried out through RNA directed DNA methylation mechanism. Characterization of diRNAs in rice and Arabidopsis were completed via CRISPR/CAS9 or TALEN triggered DSBs. Authors found 21-nt diRNAs which were created in the 35S promoter and GUS reporter in which transgene was targeted through CRISPR/Cas9. During this study authors found that unexpectedly Pol 2 transcription of the transgene was essential for better diRNA production, and for efficient diRNA accumulation which correlated the expression level of the transgene. Authors claimed the involvement of DCL4 and RDR6 in post transcriptional gene silencing which is necessary for generation of diRNAs[68].

RNA-dependent RNA polymerases (RDRs) has become a key mechanism in antiviral defense. RDR6 play an important role during defense in Oryza sativa particularly in negative-strand RNA virus RSV [41]. OsRDR6 transcript level reduced in Oryza sativa through antisense method; this reduction increases the RSV infection. Reduction of OsRDR6 stops the RSV-derived siRNAs accumulation after deep sequencing. AGO18 genetic study confirms its antiviral function which depends on its own functions to repose microRNA168 to improve the repression of rice AGO1, an essential component during antiviral RNAi [105]. miRNA168 resistant AGO1a and AGO18 expression can increase the antiviral activity in rice [37]. Authors [37] found the function of OsRDR6 in defense against RSV and RDV infection which led to the down-regulation of expression of RDR6. They find the OsRDR6 function against RDV. Results confirmed that OsRDR6 regulation by antisense OsRDR6AS which improved the susceptibility of rice against RDV infection, but OsRDR6 overexpression showed no effect on RDV infection.

Endogenous double-stranded RNA (dsRNA) nowadays known as dsRNA virus in rice Oryza sativa Endornavirus (osEV) which found in several cultivars of rice [93]. Small RNAs which were derived from osEV dsRNA were identified, demonstrating that RNA silencing distinguishes the osEV dsRNA. The quantity of osEV derived vsiRNA in OsDCL2 plants increased as
compared to the wild-type of rice. This enlarged level of vsiRNA can cause osEV unpredictability during cell division.

DCL1 plays a minor role for vsiRNA formation, and plants exhibit infection by RNA viruses [[6, 17, 21]]. It is a fact that less amount of vsiRNA is present in Arabidopsis due to loss of function of DCL2, DCL3, DCL4 triple mutant infection of Turnip mosaic virus (TMV) which leads to the suggestion that DCL1 may contain viral dsRNA substrate when all other DCL functions are conceded [32].

Figure 3: Antiviral RNA silencing in rice (Oryza sativa). Antiviral RNA silencing in rice (Oryza sativa). AGO18 adjusts AGO1 bindings to vsiRNA via sequencing the miR168 in rice. VsiRNA along with RISC target the viral RNA via translational inhibition. The action of RdR6 and cofactor SGS3 helps to produce secondary vsiRNA).
From primary transcripts of dsRNA of cauliflower mosaic virus, DCL gets excise hairpin-like structure and leads to the access of other DCLs as shown in Figure (2). (RNA silencing is originated from dsRNA which is processed into vsiRNAs through DCL2/3/4 in rice (*Oryza sativa*). AGO18 adjusts AGO1 bindings to vsiRNA via sequencing the miR168 in rice. VsiRNA along with RISC target the viral RNA via translational inhibition. The action of RdR6 and cofactor SGS3 helps to produce secondary vsiRNA).

RNA Silencing is responsible for controlling of gene expression which leads to the genome stability, stress-induced response and defense against molecular parasites in many eukaryotes [61, 81]. The formation of viral RNA and vsiRNA having double standard feature is carried out in RNA and DNA plant viruses to activate the RNA silencing [20]. Initiation, amplification, and spreading are main steps in the Virus-induced RNA silencing [96]. Silencing is started by triggering the dsRNA which is known by the consortium of Dicer-like (DCL) ribonucleases that also leads to the biogenesis of siRNA and is transformed into 21-24 nt primary vsiRNA[109]. One or more cellular RNA–dependent RNA polymerase which use ssRNA to make the perfect RNA in amplification process that can act as substrate [16], can also be used for the DCL dependent secondary vsiRNA formation. Systemic silencing is supported by vsiRNA of the secondary pool that is responsible for spreading in plants [25, 71].

Distinct ARGONAUTE (AGO)-containing effector complexes are associated by vsiRNA that also provide specificity for the DNA or RNA targets with the help of homology-dependent mechanism sequence [39, 63]. VsiRNA functional interaction which has AGO complexes with the complementary target RNAs are also responsible for cleavage and translational inhibition of the cognate RNAs [7, 53], but transcriptional repression is done by the interaction with target DNA molecule through altering DNA and histones.

**2.1.7 Multifunctional protein C1 as Silencing Suppressor:**

The systemic virus infection in the plant is the result of complex molecular interaction between the invading virus and host plant [98]. For a successful systemic viral infection, viruses must be able to produce progeny viruses in the initially infected cell, from there, they move to adjacent cells and could be transported to long distances within the plant. PD is a special intercellular organelle which crosses the cell wall to establish endomembrane and cytoplasmic continuity among the neighboring cells [66]. PDs are used by viruses for their local or cell to cell movement.
in epidermal and mesophyll cells, while the phloem allows virus for the longer distance transport to access the faraway tissues through the vascular system [18]. It is established that intercellular movement of viruses is facilitated by virus-encoded movement protein (MP) [97]. However, mode of action of movement proteins, their number and interactions with cellular structures of host varies from virus to virus.

2. Viral induction and suppression of RNA silencing

Eukaryotic organisms are dependent on genetic regulatory pathways. sRNAs are the main part of these genetic regulatory pathways which are 21-24nt in length and also have its expression inside, which further leads to precursors from double-stranded (ds) RNAs or dsRNA [88]. sRNAs show their functions from incorporation into ribonucleoprotein silencing complexes between both plants and animals from its base pairing capacity. These are involved in many functions which are post-transcriptional regulations of mRNA and its stability and also show the ability for translation, heterochromatin and transposons silencing [3]. sRNA have different proteins and different classes which are required for formation of ribonucleoprotein complexes and biogenesis that lead to important regulatory functions, its gene type regulation and other biological functions from which all are associated. Plants have different important and diverse types of sRNA and their pathways which may be important for management of multiple environmental stimuli like abiotic and biotic stresses.

It is confirmed many times that sRNA has a major role in plant-pathogen interactions. Upon infection in plants, many plant pathogens play a part for the expression of endogenous sRNA and can lead to activating the plant defense mechanisms or changing the host factor involvement [81]. RNA silencing counters many plant viruses to change the proteins like RNA silencing suppressors which may affect many parts of plant silencing machinery. In many plants, viruses show different types of symptoms which are similar to developmental irregularities and changing the organs, tissues, leaves, flowers, and fruits. These anomalies are reconcilable in which virus-induced alterations of RNA silencing-based endogenous pathways because of the direct action of VSRs in endogenous RNAs and silencing effectors, plenty of vsiRNAs in opposition to endogenous sRNAs, direct action of vsiRNAs which enter into the RNA silencing complexes and also show direct action on host genes [88]. Antisense oligonucleotides (AOs) are widely used to transmit splicing of mutation-bearing pre mRNA to inhibit it from the generation of disease-causing mRNA and compelling it to connect
into disease rescuing mRNA[84]) RNAs are used as a tool for targeting others RNAs like pre mRNA by using antisense hybridization. Vectors U1 snRNA- and U7 snRNA containing intrinsic snRNA are modified to obtain antisense sequences for targeting pre mRNA with native five ends of snRNA has been formed as shown in Figure 4 [62] [15].

**Figure 4:** Schematic pathways of Antisense oligonucleotides (AOs), Antisense RNA and RNA interference (RNAi). (A) Antisense oligonucleotides (AOs) have sequences which are complementary to exon 7. (B) Vectors U1 or U7 snRNA, can transport matched sequences to
targeting the RNAs to obtain splicing pattern. (C) RNAi mechanisms used to control pathogenic mRNA.

RNA Silencing pathways which are particularly involved in antiviral functions in plants are overviewed, and consequently, the complex interactions between host RNA and viral molecular processes will be highlighted. dsRNA production in case of viruses is the result of several types of RNAs. The majorities of plant viruses are positive (+) strand RNA viruses which accumulate several copies of (+) genomic RNA via the viral RNA dependent RNA polymerase (RdRP) through negative-stranded RNA intermediates. The perfect lengthy dsRNA molecules formed from these replication intermediates may constitute for an obvious substrate for DCLs. The vesicles or extensively rearranged intracellular membranes are associated with viral replication which is one of the common principles of (+) strand viruses, and many viral RdRP proteins are assembled with the replication intermediates. In these situations, the chances of replication intermediates of dsRNA are reduced. The great amount of vsiRNAs present in virus-infected tissues is not possible by the dsRNA replication intermediates. However, perfect vsiRNA duplexes could be generated by long, perfect dsRNAs but throughout sequencing and cloning in various plant virus systems showed that the resulting vsRNAs were not perfect duplexes [1], and solely maps the (+) strand RNA and not the (-) strand [75, 92].

The precedent observation also showed the similar accumulation of (+) strand (genomic) RNA than (-) strand (antigenomic) RNA in these viral infections. However, an extraordinary finding showed that vsiRNA from *Grapevine Fleck Virus* (family Tymoviridae) were mainly from (-) strand in its natural host. It was suggested that the main source of vsiRNAs in infected tissues is dsRNA like secondary structures than dsRNA replication intermediates within single-stranded viral RNA. Likewise, plant viruses having DNA genome (possessing neither dsRNA intermediates nor the dsRNA replication step) from RNA transcription units produce an enormous amount of vsiRNA. Indeed the chief source of vsiRNA of *Cauliflower Mosaic Virus* (genus *Caulimovirus*, CaMV) is extensive secondary structures of polycistronic 35S RNA transcript of dsDNA [70]. Similarly, RNA transcription units are derived from fold-back structures in most of the vsiRNAs associated with geminivirus infections (viruses having single-stranded genome) [9, 94].

Geminivirus is an added example in which vsiRNA originate from dsRNA molecules. Transcription is bidirectional in these single-stranded DNA viruses from dsDNA intermediates
A partial explanation of the generation of vsiRNAs is certainly when sense-antisense transcription pairs of opposite polarity overlap at their 3’ end forming and exactly corresponding dsRNAs [9].

3. Transgenic Mechanism as Silencing Suppressor

Rice (*Oryza sativa* L.), is very important cereal crop worldwide. Asia consumes 650 million tons of rice which become 90% of total world production [38], [116]. In Asia, rice production is under serious threat from 10 out of 15 most damaging viruses. In 2006-07 in South Vietnam, *Rice ragged stunt virus* (RRSV) and *Rice grassy stunt virus* (RGSV) infected 485,000 hectares of rice fields which caused 828,000 tons of rice lost worth of US$ 120 million, left millions of farmers helpless [8]. Viruses are causing enormous rice yield losses worldwide.

Genetic Engineering is most important technology for determining the resistance to field crops, whether it is found or not used by virus-derived genes that lead to the pathogen-derived resistance [79]. RNA Silencing is one of the most widely used methods to check the resistance in plants against the RNA viruses which is approximately used for more than 15 years [14, 64, 74]. RNAi mediated resistance lead to the plant virus control, or having different levels of immunity or late comings of symptoms and also sometimes no resistance occurs [5, 19, 29, 72]. Identification of [85, 86] viral genes that target the RNAi which leads to the vital role in viral infection and proliferation which can be significant at the initial stage of replication of viruses.

It is very important to control rice damaging viruses to ensure the continuity of world population and global food safety. Tenuiviruses have thin filaments with 3 to 10 nm diameter; they have 4-6 single-stranded segments of RNA having positive (sense) and negative (antisense) polarization with or without one nucleocapsid protein [89]. The genomes of RHBV and RSV are comprised of 4 single-stranded segments of RNA, they have seven genes and are designated in decreasing molecular weight order. The negative polarity is found only in the first segment of RNA while the remaining three are antisense. The genome of RGSV has six segments of ssRNA which are all antisense and contain 12 genes. The viral genes are transcribed into mRNAs by the mechanism of cap-snatching[83].

Reoviruses which are plant-infecting viruses are found in the genus Oryzavirus, Fijivirus and Phytoreovirus and family Reoviridae [51]. Their vectors are planthoppers or leafhoppers which transmit them in a propagative and persistent manner. Insect and plant cells infected by Reoviruses have a common feature in which virus particles are found in the tubules. One of the
Fijivirus, *Rice Black-Streaked Dwarf Virus* (RBSDV) replicates in the plant as well as its vector (invertebrate insect) [30]. The genome of the virus comprised of 10 segments which are double-stranded RNAs (dsRNAs), which are labeled as S1 to S10, as they occur in the electrophoretic pattern of migration on polyacrylamide gels like SDS.

Recently two groups have proposed a tentative species named *Southern Rice Black-Streaked Dwarf Virus* (SRBSDV) in the Fijivirus genera [123, 129]. The spread of SRBSDV throughout the northern Vietnam and southern China has been rapid and caused serious damage in certain rice growing regions [99]. Transmission of this virus occurs by a white-backed planthopper (*Sogatella furcifera*; order *Hemiptera* and family *Delphacidae*), in a manner which is persistent and propagative. SRBSDV is related very closely to RBSDV because both viruses have nearly 81% identity of amino acids in P7-1, it is encoded by S7 genomic segment [129]. Moreover, the infection of SRBSDV also induces the development of tubules in the cells of insect vectors and plants. Nevertheless, the origin and significance of these tubules remained unknown. In this study, it was identified that P7-1 of SRBSDV has the main ability to form tubules which grow from the surface of the cell when other viral proteins are absent.

In various cellular processes, Protein-Protein Interactions (PPI) plays an important role. Characterization and identification of PPIs could provide important insights into protein functions helpful at the cellular level [121]. The mapping of viral protein interaction network will significantly help in understanding the cycle of virus infection, viral pathogenicity mechanism, and host-virus interactions. It has been reported that Intra-viral PPIs does occur in *Rice Streak Virus* (RSV) and *Southern Rice Black-Streaked Dwarf Fijivirus* (SRBSDV) [45, 47]. It can be easily assumed like in a lot of viruses that intro viral PPIs could play an important part in the replication cycle of RSV and SRBSDV. Therefore, it will be interesting to investigate whether PPIs exist among RGSV specific proteins-proteins shared with RSV and whether these PPIs are conserved in the members of genus Tenuivirus. Among the Tenuiviruses RSV is a thoroughly studied virus.

It has caused widespread losses in rice production in Japan, Korea, and China [23, 105]. There are reports that it not only infects rice but wheat, maize, oat, foxtail, Arabidopsis, millet, and weeds as well [46, 90]. At the moment it is identified that various host factors interact with RNA or proteins of RSV, which are being manipulated by RSV for the completion of its life cycle [58, 128]. Remarkably, P2 the nonstructural protein of RSV, a viral silencing suppressor, has the
ability of binding to rice host protein which is similar to the suppressor of gene silencing in Arabidopsis (AtSGS3). Formerly it has been found that P3 which is a nonstructural protein of RSV encoded by RNA3 acts as RNA silencing suppressor [111]. It was hypothesized by Wu and his fellows that dual function is played by a P3 protein, both in the facilitation of viral infection and inhibition of pathogenic development by inducing host defense mechanism [102].

Eukaryotes and plants use a much-conserved mechanism RNA silencing as a defense strategy against transposons and viruses [104]. Generally, dsRNAs trigger RNA silencing. Cellular specific enzymes recognize the dsRNAs and chop them into interfering RNAs of small lengths of 20-24 nucleotides. The siRNAs plays the key role as a guide by blocking expression of genes at transcription, post-transcription and translation levels by forming a silencing complex [26, 31]. Many viruses in their life cycles produce dsRNAs which means viruses can be the targets as well as initiators of RNA silencing [115]. As a counter defense strategy, many plant viruses suppress the RNA silencing by encoding a protein [20, 95].

Various studies have witnessed that Viral Suppressors of RNA silencing (VSRs) target particular steps of RNA silencing: since the synthesis of dsRNAs until the integration of siRNAs into the RNA Induced Silencing Complex (RISC) [43, 76]. Additionally, some VSRs also interfere with the movement of signals for silencing. Identification of some VSRs has been identified from the animal as well as plant viruses [43, 124]. Most of the plant viruses have only one VSR, but some plant viruses encode more silencing suppressors, which could target the main steps of RNA silencing [34, 59].

4. Conclusion

In this review, we have tried to summarize some report cases representing successful gene silencing. Some cases have investigated the gene silencing which has direct interaction for virus transmission. Expression of siRNA molecules in rice plant for insect feeding and inducing silencing will be the best method for RNAi-based pest control.

South, Southeast and East Asian countries are severely attacked by rice viruses and lead to reduced s production of rice in these countries. The use of Insecticides to manage the insect vectors is the main approach, but it increases the cost of production which is the main burden on rice farmers. Genetic resistance manipulation for rice viruses is the most economical approach to manage them.
Recently, an important antiviral vsiRNA-program which is named as AGO-containing complex is capable of leading directed sequence-specific cleavage, somewhat translational inhibition and also transiently express the sensor construction which contains viral segments is also reported. The use of RNAi is a prominent and favorable method to maintain the resistance to rice viruses. The mapping of viral protein interaction network will significantly help in understanding the cycle of virus infection, viral pathogenicity mechanism, and host-virus interactions. The development of transgenic rice plants which have the extra high capacity to maintain resistance against viruses, targeting of viral genes which have a special role in virus entry and proliferation at the start of viral replication. As a much-conserved mechanism, RNA silencing is used by eukaryotes and plants as a defense strategy against transposons and viruses. Classical virus control methods have partial success at reducing the destruction caused by plant viruses. The discussed data highlights the discovery and application of vsiRNA against virus attack and supports the notion of utilizing some novel germplasm for the increment of future rice productivity.

Engineering plants for silencing point of view can be a most effective method to control the persistent viruses by silencing of genes which have an important role in transmission. Developing the transgenic plants for induction of silencing in vectors will be innovative by developing specifically expressed genes in insects.

**Abbreviations**


**Acknowledgements:**

We are extremely sorry for not citing all the related studies in this review because of space limitation, although we have cited some up-to-date research about RNA silencing. We are
thankful to Prof. Wei Taiyun (College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China) for critical reading of this review.

**Author Contributions:** All authors have read and approved the final manuscript.

**Conflict of interest:** All authors have no institutional or financial competing interests. All authors declare no conflicts of interest.

**Research involving human participants or animals:**

No part of this study was performed with human participants or animals by any of the authors.

**Funding:**

This work was supported by Fujian Agriculture and Forestry University, Fuzhou, Fujian, China, Science Fund for Distinguished Young Scholars (xjq201622).

References


