RNA interference (RNAi) mechanism is a natural process occurring in both plants and animals which silence the specific genes before being translated. RNAi is the key biological process in plants and has many different forms including transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS). Plants can uptake and process externally applied double stranded RNAs, hairpin RNAs and small interfering RNAs prepared to silence genes of viruses, fungi and insects. The exogenously applied small RNAs move locally and systematically and induce RNAi mediated resistance. Understanding the plant response and processing of small RNAs could result in development of novel biotechnological approaches. In this review we analyze the biogenesis and function of endogenous set of small RNAs, exogenously applied small interfering RNAs and their response in plant cells.

**Keywords**
RNAi, dsRNA, endogenous RNA, Exogenous RNA, siRNA

**Abstract**
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Introduction
The phenomenon of RNA interference was first discovered in a nematode worm *Caenorhabditis elegans*, where double stranded RNA (dsRNA) bring about specific gene degradation of mRNA which results in gene silencing. RNAi mechanism in plants gives different types of small RNAs (sRNAs) including siRNAs, miRNAs, ta (trans-acting)-siRNAs, and pha (phased)-siRNAs. siRNAs are generated from various Double stranded RNA molecules(dsRNAs) i.e., dsRNA derived from RNA virus replication, dsRNAs synthesized by plant endogenous RNA-dependent RNA polymerase, dsRNAs of natural sense and antisense transcripts, and single-stranded RNAs that form hairpin-loop secondary structures (Baulcombe 2004; Voinnet et al., 1999; Rosa et al., 2018). These dsRNA molecules are recognized and cleaved by Dicer like proteins present in the cell to generate siRNAs. After cleaving of dsRNA into siRNA, the siRNA duplex molecules move into RNA-induced silencing complex (RISC), where Argonaute (AGO) protein plays a major role. The RISC complex cleaves the siRNA duplex, where one strand retains and serves as guide strand. The guide strand serves as a recognition molecule to identify the cognate mRNA molecule which is translating into protein. The guide strand
recognizes the mRNA strand by Watson-Crick base pairing, leading to RISC cleavage/slicing of RNA target. Plants encode multiple AGO proteins, the detailed description of evolution and function of different AGOs can be found in the review of Zhang et al., 2015. Among all the Argonaute proteins AGO2 has been reported to be involve in siRNA-related antiviral, antibacterial and anti-stress defenses (Jaubert et al., 2011). AGO protein contain three major domains like PAZ, MID, and PIWI domains. After slicing the small RNA target duplex is separated by variable domain (ND) present at the N-terminal region of AGO proteins. The PAZ domain, which is close to ND bind the 3’ end of small RNAs (guide RNA) (Lingel et al., 2004). MID and PIWI domains are present in C-terminal domain. The junction of these two domains contains a pocket which anchors the 5’ end of the guide RNA. The siRNA- target duplex loaded AGO protein cleaves the target RNA by PIWI domain, which contains a RNase-H-like structure and a conserved catalytic site (Parker 2010). By the action of RNase-H the target RNA is cleaved and degraded. By this functional mechanism the target gene or protein is degraded without undergoing to translation thereby disrupting the central dogma of life.

Endogenous small RNAs

Small RNAs or micro RNAs control the gene expression mechanism by silencing the target mRNAs without encoding into protein. In plants small RNAs perform many functions such as to control expression of genes encoding transcription factors, stress response proteins and other proteins which are involved in the growth, development and physiology of plants (Rogers and Chen, 2013). Small RNAs are mainly classified into micro RNAs (miRNAs) and small interfering RNAs (siRNAs) (Parent et al., 2012). Both miRNA and siRNA are derived from double stranded RNA molecules. miRNAs are derived from the hairpin loop precursors from the single double stranded RNA molecule (dsRNA) whereas siRNAs are derived from the double stranded duplexes of the two RNA molecules. The first miRNA lin-4 was discovered in Caenorhabditis elegans in the year 1993 by Victor Ambros and his colleagues.

miRNA pathway

In plants miRNA biogenesis begins in nucleus with the generation of long (>70nt) bulged hairpin RNA known as primary microRNA (pri-miRNA). In plants miRNAs are derived from the non coding regions (Introns) and few of them are located in 5’ UTR or 3’ UTR regions of a nuclear gene and animal miRNAs are encoded from the introns of protein coding genes. Plant miRNAs are transcribed by RNA Pol II which produces capped and poly-adenylated long primary miRNA transcripts (pri-miRNAs), these are then processed into precursor miRNA (pre-miRNA) of about 80-500 nucleotides in length, by an enzyme, DICER-LIKE 1 (DCL 1), with RNase III activity (Kurihara et al., 2006). A part of pri-miRNA forms hairpin loop like structure by folding which is cropped by DCL 1 complexes. The DCL 1 enzyme complexes cut the pri-miRNAs at a distance of 16-17bp from the miRNA duplex in the nucleus (Zhu et al., 2013). The conversion of pri-miRNA to pre-miRNA requires various proteins like double-stranded RNA (dsRNA)-binding protein Hyponastic Leaves1 (HYL1) and C2H2 zinc finger protein SERRATE (SE) along with other factors like CBC, DDL, and TOUGH (Yang et al., 2006; Parry et al., 2007). These proteins are present near the nuclear processing centers called D-bodies or SmD3-SmB-bodies (Kurihara et al., 2006). Pre-miRNA is unstable in nucleus and processed into miRNA-miRNA duplex by DCL1 and
stabilized by S-adenosyl methionine dependent methyltransferase HUA ENHANCER 1 (HEN 1), which methylates all plant silencing RNAs. The mature miRNA duplex are then exported to cytoplasm by the action of HASTY protein (an ortholog of exportin-5 protein) (Bollman et al., 2003). According to their position on the hairpin-shaped precursor the miRNA and miRNA* are named as miRNA-5p and miRNA-3p respectively (Kozomara et al., 2014). The produced miRNA-5p or miRNA-3p are methylated by HEN1, which protects from being degraded by small RNA degrading nuclease (SDN) class of nucleases (Ramachandran and Chen 2008). Finally the functional miRNA will be incorporated into Argonaute protein containing RNA Induced Silencing Complex (RISC). Plant miRNAs associate with AGO1 protein to induce post-transcriptional gene silencing (PTGS) resulting in RNA silencing or translation inhibition (Li et al., 2013).

Trans-acting siRNA pathway

In plants, a group of microRNAs (miRNAs) induce the production of class of small interfering RNAs (siRNAs) which lead to gene silencing are called trans-acting siRNA (ta-siRNA) (Jacobs et al., 2016). The pathway for trans-acting RNA is a combination of both miRNA and siRNA pathway but have a different mechanism. Like miRNA, ta-siRNAs are 21 nucleotides long and are derived from the intragenic regions of the nuclear gene. In ta-si RNA biogenesis, a single stranded RNA transcript is first cleaved by AGO1 protein directed by miRNA. The cleaved product is converted into long dsRNA molecule by RDR6, which is then processed by DCL4 into 21-nt siRNAs with 21 nucleotides phasing starting from the mi RNA cleavage site (Xie et al., 2005). For the production of ta-siRNA in Arabidopsis suggests that 22 nucleotides size of miRNA is sufficient to induce ta-siRNAs, whereas 21-nt of miRNA are not (Chen et al., 2010). Like miRNAs methylation, the ta-si RNA molecules are methylated by HEN1 and interact with AGO1 or AGO7 to direct the degradation of target mRNA. In Arabidosis, ta-siRNA affect the auxin response involved in phase transition from initial juvenile stage to reproductive stages (Fahlgren et al., 2006). A large number of ta-siRNA like siRNAs are collectively known as phased siRNAs or phasiRNAs. PhasiRNAs have been identified in Arabidosis, tomato (Shivprasad et al., 2012), tobacco (Li et al., 2012), rice (Song et al, 2012) and in many other crops. RNA sequence from protein-coding genes (Exons) and non-coding genes (introns), transposons and repetitive DNA can serve as a template for phasiRNA production. PhasiRNAs are derived from the Leucine rich Repeat (NB-LRR) disease resistance genes and play a major role in controlling the stress proteins and defense related genes (Fei et al., 2013).

Exogenous small RNAs

Doule stranded RNA (dsRNA) act as a signal molecule to induce RNAi in plants and higher organisms. When a dsRNA molecule corresponding to endogenous mRNA is introduced into the cell of the organisms, the cognate mRNA is silenced. This phenomenon was discovered in Caenorhabditis elegans where dsRNA corresponding to specific gene injected in to worm the corresponding gene was disappeared (Fire et al., 1998). A dsRNA molecule of 21nt long could trigger RNAi in human cells without inducing interferon response was reported by Elbashir et al., In a study conducted by Elbashir et al., synthetic siRNAs are constructed to mimic dsRNA RNAi trigger in eukaryotic cells. Plants can uptake synthetic dsRNA or siRNAs when exogenously applied and also capable of entering in to vascular bundles and distant
SiRNAs are synthesized commercially using solid phase synthesis. In vitro transcription can also be used to synthesize siRNAs or short hairpins. Exogenously applied siRNA can induce silencing as that of miRNA pathway, the difference is that exogenously applied dsRNA already mimic the product of Dicer. siRNA sequences are designed based on Watson-Crick base pairing complementarity to the target mRNA. As a result siRNA-mediated RNAi result in cleavage of target mRNA by the slicer activity of Ago2 protein. Finally the target mRNA is degraded and cleaved. Short hairpin RNAs (shRNAs) are small RNAs which are structurally similar to siRNAs and miRNAs. Like siRNAs, the guide strand and the passenger strand are perfectly complementary to each other. shRNAs are designed to function like that of both miRNA and siRNA. shRNAs can be synthesized artificially and can be transfected into cell like siRNAs. shRNAs can be expressed from plasmid DNA or viral vectors in the eukaryotic cells. The small RNAs can be applied onto plant leaves by mechanical inoculation, spray application, loading on nano clay sheets, materials that promote RNA adhesion, spreading by pipettes or brushes and root soaking (Dubrovina and Kiselev, 2019).

**Induction of dsRNA in plants transgenically**

Double stranded RNA (dsRNA) derived from transgenes of sense and antisense RNA homologous to coding gene can induce RNA interference (RNAi) mechanism in plants. Plants engineered with dsRNA gives specific and robust resistance against plant viruses through RNAi. Transgene based RNAi approach was firstly used in plant virus disease control. hpRNAs have been widely used to silence the target genes and viral RNAs in plants (Watson et al., 2005). For the host delivery of RNAi, plants are genetically modified to express dRNA molecules with sequence derived from the target gene. RNAi constructs are made by cloning a fragment of target gene coding sequence in sense and anti sense orientation, separated by intron/spacer region, under the control of a constitutive or tissue-specific promoter. Upon transcription, sense and antisense strands of the target gene complement each other and form an intron spliced hairpin RNA of which a significant portion is dsRNA (Waterhouse and Helliwell, 2003; Dutta et al., 2015). Most researchers have constructed stable transgenic plants in which the transgene constructs generate double-stranded (ds) RNA, either directly or indirectly. The dsRNA is then processed to siRNA in vivo by Dicer which is an endogenous enzyme present inside the cell. Viruses can also be used to deliver the silencer sequences using a transient silencing protocol. Both of these basic protocols use Agrobacterium tumefaciens to deliver transgene constructs into plant hosts (Yin et al., 2005). Compared to the application of chemicals, transgenic plants are cost effective and a promising tool. While there are legislative limitations on cultivation of transgenics in many countries, the information on consequences of genome modification are insufficient and raises public concerns on safety of genetically modified organisms (Dubrovina and Kiselev, 2019). Virus resistant plants are generated by RNA silencing mechanism by expressing virus coat protein genes. Transgenic papaya plants were generated against *papaya ring spot virus*, coat protein gene isolated from *papaya ring spot virus* was cloned into a binary vector with 35S promoter and transformed into somatic embryos of papaya cultivar by microrocketile bombardment. The transformed transgenic lines of potato showed resistance against *papaya ring spot virus* due to RNA mediated interference (Kerbundit et al., 2007). Transgenic plants of potato expressing
hpRNA construct targeted against plasma membrane-localized Syntaxinrelated 1 (SYR1) showed enhanced resistance to the oomycete pathogen *Phytophthora infestans* (Eschen-Lippold *et al.*, 2012).

**Methods to induce RNAi in plants**

An important aspect to induce RNAi in plants is to deliver the molecules of silence inducing dsRNA or hpRNA. RNA can be delivered by transforming plants with transgenes that encode hpRNAs or viral RNAs. The most commonly used methods for delivery of dsRNA in plants cells are agroinfiltration, microbombardment and virus induced gene silencing (VIGS).

**Agroinfiltration**

Agroinoculation or Agroinfiltration is the process of delivering active DNA molecules by using transformed *Agrobacterium* cells into plant cells. When *Agroacterium* is injected into the cell it transfers the T-DNA plasmid and integrates in the nucleus of the host cell. The genes of the T-DNA express when they are introduced into the cell. When the leaves are infiltrated with *Agrobacterium* culture containing T-DNA region with a transgene that encodes an endogenous gene will trigger the RNAi mechanism. The infiltration of hairpin constructs are very effective, because the dsRNA is directly processed into siRNA (Johansen *et al.*, 2001). Leaf infiltration of *Agrobacterium* is the popular method for transient gene expression in *Nicotiana benthamiana* (Goodin *et al.*, 2008). Agroinfiltration method has been successfully employed in *Arabidopsis* (Wroblewski *et al.*, 2005) and in many other crops like Tobacco (Yang *et al.*, 2000), Tomato (Orzarez *et al.*, 2006), Grape vine (Santo-Rosa *et al.*, 2008) etc. Agroinfiltration provide a reliable and convenient way in achieving level of gene expression like inducing resistance to crown gall disease in apple (Dunoyer *et al.*, 2006), OsGEN-L-green fluorescent (GFP) fusion protein in rice (Moritoh *et al.*, 2005) etc.

**Microbombardment**

In this method the DNA is transferred into the nucleus of the cells by nuclear bombardment. Plant tissues are bombarded with gold or tungsten particles that have been coated with DNA. Plant is a routinely approach for studying the gene expression in monocotyledonous plants including rice (Christou, 1997). Cells bombarded with particles containing dsRNA, siRNA or hpRNA trigger the RNA silencing pathway in plants. A repressor of GUS protein was silenced in transgenic tobacco (*Nicotiana tabacum*) plant using micro bombardment of dsRNA was reported by Klahre *et al.*, 2002.

**Virus Induced Gene Silencing (VIGS)**

Virus Induced gene silencing is a reverse genetic tool, which uses viral vectors to produce dsRNA which induce RNA mediated gene silencing in plants. VIGS exploits the innate plant defense system of Post Transcriptional Gene silencing against intracellular viral replication and proliferation (Lange *et al.*, 2013). Most of the plant viruses contain RNA as their genetic material. When a virus infects the cell the RNA is released upon the disassembly of Coat protein. The RNA is then replicated by the virus encoded RNA dependent RNA polymerase enzyme to produce sense and anti-sense strand RNA i.e dsRNA. The viruses which are capable to produce the dsRNA trigger the RNAi response in plant cells. The viral genomes are modified such that viral RNAs are generated by invitro transcription and are placed in the left and right borders of T-DNA plasmid region under the control of CaMV 35S promoter (Liu *et al.*, 2002). The construct is
delivered into plant by Agroinfiltration and can produce viral infection triggering the RNAi response. Many of the viral vectors are derived from the single stranded RNA viruses like Potato virus X (PVX), Tobacco mosaic virus (TMV) and Tobacco rattle virus (TRV) (Purkayastha and Dasgupta 2009). As compared to RNA viruses, DNA viruses have not been commonly used as expression vectors because of their size constraints in their movement (Kjemtrup et al., 1998). Expression vector derived from the Turnip Yellow Mosaic virus (TYMV) has ability to induce VIGS in Arabiopsis thaliana (Pflieger et al., 2008). Several monocot plants are successfully targeted by VIGS like BSMV and BMV in Hordeum vulgare (Holzberg 2002), BMV and RTBV in Oryza sativa (Purkayastha et al., 2010), BSMV in Triticum aestivum (Scofield 2005) and BMV in Zea mays (Ding et al., 2006).

**Induction of dsRNA in plants non-transgenically (RNA based vaccine)**

RNA silencing is thought to play an important role in the protection against invading nucleic acids in plants towards its application as a result (Bartel 2004; Baulcombe 2004). External application of invitro synthesized or bacterially produced dsRNA showed resistance against viruses, invading pathogens and insect pests. The exogenously applied dsRNA spread locally and systematically to phloem bundles and to distant cells and induce RNA interference-mediated plant pathogen resistance. Many studies reveal that exogenously applied RNA molecules are capable of affecting the mRNA of target genes which leads to mRNA degradation in plant pathogens. For the topical application of dsRNA in a non-transgenic manner, dsRNA are expressed either by using invitro or invivo production systems with appropriate expression vectors (Robinson et al., 2014). The vector elements required for RNA transcription are T7 or T3 DNA-dependent RNA polymerase (DdRp) promoters and a multiple cloning sites for insertion of sequence to be transcribed. For the production of dsRNAs, two invitro systems are available to induce virus resistance. Studies conducted by Tenllado and Diaz-Riaz 2001 used invitro single stranded RNA (ssRNA) transcription system for the production of dsRNA for topical application. In the invitro transcription system, they have transcribed by using two DNA plasmids each encoding a sense and anti-sense complementary DNA (cDNA) sequence from the respective virus was transcribed into ssRNA by T7 DdRp and the respective sense and antisense ssRNA subsequently annealed. In the second invitro system, *Pseudomonas syringae* dsRNA bacteriophage phi 6 (ϕ6) RNA dependent RNA polymerase (RDR) is used for the generation of full length dsRNA from ssRNA templates (Aalto et al., 2007). In this system the insert is transcribed by T7 DdRp to produce the ssRNA template strand for the RDR of ϕ6 that subsequently uses the transcribed ssRNA to generate the complementary strand within single plasmid vector in a *E.coli* bacteria. For the large production of dsRNAs invivo expression systems were used which uses E. coli strain HT115 (DE3). A study conducted by the Timmons et al., 2001 to silence the endogenous gene of *Caenorhabditis elegans*, they have used E. coli strain HT115 which harbours the pro-phage λDE3 encoding the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 polymerase gene for dsRNA transcription. IPTG acts as inducing molecule to activate the transcription and to produce dsRNA. The dsRNA derived by this method are very efficient and can be produced in large amounts (Gan et al., 2010). dsRNA sequences derived from the viral sequences and introduced into tobacco plant by agro-inoculation showed resistance against three virus groups namely tobamovirus, potyvirus.
and alfamovirus group (Tenllado and Díaz-Ruiz, 2001). Some more examples for non-transgenic based RNAi approach are listed below (Table 1):

**Table 1** Report on Induction of dsRNA to plants non-transgenically (RNA based vaccine)

| Crop                          | Virus                                          | Virus derived region                      | Observation Recorded                              | Reference                     |
|-------------------------------|------------------------------------------------|-------------------------------------------|---------------------------------------------------|-------------------------------|
| N. tabacum cv. Xanthi, C. chinense | PMMoV                                          | Virus replicase gene (977 bp)             | Significant reduction of PMMoV virus in tobacco plant | Tenllado and Diaz-Ruiz, 2001 |
| Bhut Jolokia                  | Cucumber Mosaic Virus                          | CMV-2b                                    | Disease incidence ranged from 0 to 29% in dsRNA treated plants in contrast to 55 to 92% when only CMV was applied | Borah *et al.*, 2018         |
| Sesbania grandiflora          | Sesbania mosaic virus (SeMV)                   | Coat protein (CP) and (Movement Protein)  | dsRNA of CP and MP conferred 53% and 64% resistance respectively | (Konakalla *et al.*, 2019)   |
| Tobacco                       | Tobacco Mosaic Virus (TMV)                     | p126 and CP                               | dsRNA of p126 and Cp conferred 65% and 50% resistance respectively | (Konakalla *et al.*, 2016)   |
| Cucurbits-Cucumber Watermelon and Squash | Zucchini Yellow Mosaic Virus (ZYMV)          | Helper Component Proteinase (HC-Pro)     | exogenous dsRNA application in cucumber, watermelon and squash plants, dsRNA HC-Pro conferred resistance of 82%, 50% and 18%, and dsRNA CP molecules of 70%, 43% and 16%, respectively. | Kaldis *et al.*, 2018        |

**Barriers in foliar uptake of dsRNA**

Double stranded RNA (dsRNA) when sprayed on plants produce an elicitor response to induce RNA interference RNAi. Foliar application of dsRNA should be in sufficient number to reach the target cells of the plant. The dsRNA sprayed must travel the surface of the leaf, through cuticle, to cross through the cuticle, cell wall and plasma membrane to gain entry into cell’s RNAi machinery. Once it enters into cell, the dsRNA move to adjacent cells through plasmodesmata and to distal cells by phloem vessels. Small dsRNA generated in the cell’s machinery can move through symplastically into phloem vessels and to distant cells. There are many barriers in the efficient delivery of dsRNA to the target cells (Bennett *et al.*, 2020).

**Cuticle acting as a barrier**

Cuticle layer composed of lipids and hydrocarbons covering the epidermis of leaves, young shoots and fruit. Cuticle is known to impede the absorption of exogenous
water and solutes. The cuticular properties make it resistant to dsRNA absorption given the water solubility of dsRNA. To test this fluorescently labeled (Cy3) 21 base pair (bp) siRNA was applied on the adaxial surface of Palmer Amarnath (Amaranthus palmeri) with 0.5% of spreading surfactant Silwet L-77. Treated leaf samples were collected after 4hrs of application and observed under fluorescence microscopy. It was observed that most of the applied dsRNA was found on the adaxial surface itself. The fact that Cy3-SiRNA remained on the leaf surface due to its high molecular size and relatively water soluble molecule (Schreiber, 2005).

To increase the cuticle penetration of siRNA and to achieve RNAi phenotypes abrasion, high pressure spraying and abaxial stomatal flooding has been suggested. Spraying particles like celite, alumina of sizes >2 microns with or after siRNA application at pressures <700 kPa resulted in improved cuticle penetration of the siRNA and resulted in robust visual RNAi phenotypes (Huang et al, 2018).

**Nuclease stability as a Barrier**

In vivo nuclease degradation is known to impact the efficiency of applied siRNA to elicit an RNAi response in mammalian systems (Behlke, 2008). Very less information is known about the impact of nuclease on delivery efficiency of siRNA in plants. It was suggested that nuclease degradation could impact the ability of foliar applied siRNA to gain access to plant cell (Pérez-Amador et al., 2000). An experiment was conducted to investigate the impact of nuclease on siRNA activity where 22bp siRNA was applied by syringe infiltration to an leaf of N. benthamiana. The 22 bp siRNA was infiltrated in the presence or absence of nuclease inhibitor or the cationic polymer polybrene. Leaf tissue samples were collected from infiltration site at 0, 1, 2, 4, 6, 8, and 24h post application. The results were analyzed using anionic exchange HPLC. It was observed that when siRNA applied in absence of nuclease inhibitor or polybrene was not detectable at 6 h post application whereas siRNA applied with a nuclease inhibitor or polybrene which binds dsRNA still detectable at 24h application (Schreiber, 2005). The results suggest that nucleases act as a barrier to efficient dsRNA delivery.

**Response activated by dsRNA in Plants**

Plants have evolved with a variety of immune systems like Pathogen Associated Molecular Patterns (PAMPs)- triggered immunity (PTI), Effector Triggered Immunity (ETI). When a pathogen infects the plant the immune systems are activated and employ resistance (R) proteins to activate ETI. The pathogen avr genes react with R proteins which is called R mediated gene resistance leading to Hyperensitive response (HR). the first R gene conferring resistance was identified as tobacco N gene in *Tobacco Mosaic virus* and Avr gene was identified in the replicase gene (Whitham et al., 1994). Unlike fungal and bacterial pathogens viral genomes replicate within host leads to RNA silencing pathways and play a critical role in anti-viral defense. During the replication of the RNA/DNA viruses dsRNA is the common molecule and act as an intermediate. Upon the recognition of dsRNA molecule in plant RNA silencing and Pathogen Triggered immunity gets activated in the plant (Niehl et al., 2018). The other responses that are induced by viruses and dsRNA in plants include the production of reactive oxygen species (ROS), induction of hormone signaling, activation of mitogen activated protein kinases and induction of defense gene expression (Nicaise and Candresse, 2017). The anti-viral immune response is mediated by Leucine-Rich-Repeat
receptor like kinase (LRR RLK) NIK1. Upon activation of NIK1 during infection, a signaling cascade leading to down regulation of gene associated with translational machinery and eventually the down regulation of viral and hosts mRNA translation, is induced.

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