Journal of Cell and Molecular Research (2018) 10 (1), 40-46 DOI: 10.22067/jcmr.v10i1.70766

Review Article

MicroRNA-mediated Resistance to Plant Viruses

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Received 4 February 2018 Accepted 19 September 2018

Abstract

MicroRNAs (miRNAs) are 20-24 nucleotide small RNAs which are processed from nuclear-encoded transcripts. miRNAs control the expression of target transcripts by cleaving or translational inhibition of the target RNAs. Artificial microRNAs (amiRNAs) are modified endogenous miRNA precursors in which the miRNA: miRNA duplex is replaced with sequences to silence a target gene. amiRNAs are used as new transformation techniques in eukaryotes and have been proven to be more effective in specificity and stability than other RNA-mediated gene silencing methods. amiRNA-based antiviral defense is an effective and new approach to engineer resistance to plant viruses. Here, we summarize the role of miRNAs in resistance to plant viruses.

***Keywords*:** amiRNA, Gene silencing, miRNA, Plant viruses, Resistance

miRNA History

The first report on microRNAs (miRNAs) dates back to 19 years ago when Lee et al., screened the *Caenorhabditis elegans* genes to study the development of its larvae. *lin-4* gene was shown to control the timing of larval development. Further studies indicated that *lin-4* RNA binds to 3' untranslated region of *lin-14* mRNA and blocks its translation. This function of lin-4 is part of the regulatory pathway which triggers a transition from L1 (larval stage 1) to the L2 stage (Lee et al., 1993; Wightman et al., 1993). miRNA genes have been found in mammals, fish, worms, flies, plants and many other eukaryotic organisms (Bartel, 2004).

Pre-miRNA Transcription

miRNA encoding genes are located on non-coding parts of the genome. Plant miRNA genes are gathered together in the form of gene families.

miRNAs are long strands (sometimes even longer than 1000 nucleotides), which are bending on themselves and forming stem-loop structures. They are capped and polyadenylated at 5' and 3' ends, respectively. These features indicate that plant miRNA genes are probably transcribed by RNA Polymerase II (Devers et al., 2011; Tanzer et al., 2008).

miRNA Processing

Plants and animals differ in miRNA synthesis at the post-transcriptional stage. The main step in miRNA maturation process is cleaving the mature miRNA from its precursor. In animals, this is performed by Drosha and Dicer enzymes. Plants have a 'Dicer-like' protein, DCL1, analogous to Dicer and Drosha, that generates the double-stranded miRNA: miRNA (Tanzer et al., 2008; Bazin et al., 2012; Wang et al., 2007). HYPONASTIC LEAVES1 (HYL1) protein also appears to contribute to DCL1 in plants. The HYL1 protein helps DCL1 to recognize cleavage site. The next step in the maturation process of plant miRNAs is methylation of the 3' end of "miRNA: miRNA" double-stranded molecules by HUA ENHANCER1 (HEN1) in the nucleus. Methylation of this molecule prevents its degradation (Kai and Pasquinelli, 2010). After cleaving by DCL1 and methylation by HEN1, "miRNA: miRNA" double strands are transferred from the nucleus into cytoplasm via a Ran-GTP-dependent mechanism, by HASTY (HST), a member of importin β family of nucleocytoplasmic transport receptors (Figure 1) (Jones-Rhoades et al., 2006; Mendes et al., 2009).

RNA-induced Silencing Complex (RISC)

The synthesis stage is the formation of double-stranded "miRNA:miRNA" molecules in the cytoplasm. A helicase breaks the bonds between the two strands of the molecule and separates them. The "guide strand" of a miRNA is incorporated into silencing complex and enables target recognition via complementary base pairing (Wang et al., 2007; Kai

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and Pasquinelli, 2010; Jones-Rhoades et al., 2006; Mendes et al., 2009; Hammond et al., 2007). RISC has an affinity for strands with a less stable 5' end. "Passenger strands" are often degraded (Thomas et al., 2010; Winter et al., 2009), although recent findings indicate that some of them may play an important role in regulating gene expression or triggering silencing mechanisms (Fullaondo and Lee, 2012).

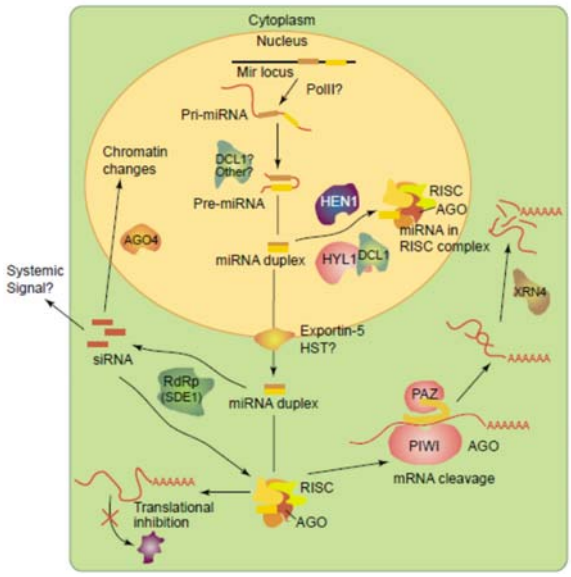

Figure 1. miRNA biogenesis and its regulation mechanisms in plants. Transcription of miRNA by RNA polymerase II produces a pri-miRNA of about 1 kb. This pri-miRNA is cleaved into pre-miRNA by DCL1 and other proteins to yield the fold-back precursor pre-miRNA. Duplex miRNAs are exported to the cytoplasm out of the nucleus by HST (Exportin-5). These proteins process the pre-miRNA to form a miRNA–miRNA duplex, which is loaded on the RISC. The RISC complex is guided by the miRNA to target mRNAs. The cleaved target mRNAs are degraded by XRN4 protein. Secondary small interfering RNAs (siRNAs) are generated from the action of RNA-dependent RNA polymerase (RdRP). The RISC can also cause translational inhibition (Kidner and Martienssen, 2005).

Translation Repression

The RISC-miRNA complex can repress translation of the target RNA in two ways. In animals, miRNAs often repress translation of their target RNAs by an imperfect sequence which is matched with 3' untranslated region (UTR) (Sontheimer and Carthew, 2005). After binding to 3' UTR, miRNAs can prevent translation of mRNAs by interfering with translation initiation factors or disrupting poly(A) tail's function (Gu and Kay, 2010). It has also been shown that RISC may form a stable complex with polyribosomes and prevent translation, even after its initiation and at the elongation phase (Bartel, 2004).

mRNA Degradation

Whether a miRNA represses translation or degrades its target RNA, depends on the degree of complementarity between their sequences (Lu et al., 2008). It has been observed in plants and mammals that a nearly perfect base complementarity between miRNA and its target RNA leads to degradation of the target rather than repressing its translation (Bartel, 2004; Kadotani et al., 2004; Nakayashiki, 2005). A perfect base complementarity appears to be required for effective mRNA cleavage by miRNAs (Wang et al., 2007). MiR-172 has been shown to act as a translation repressor in *Arabidopsis* (Sontheimer and Carthew, 2005). As soon as the target is effectively cleaved, the miRNA molecule is able to cleave other target RNAs or direct them toward mRNA processing bodies to be subsequently degraded (Bartel, 2004).

Transcriptional Gene Silencing

miRNAs turn off genes at transcription level by methylation of chromatin in plants. The effects of the level of sequence complementarity between miRNAs and its target RNA on the degree of RNA methylation is not yet clear (Jones-Rhoades et al., 2006).

RNA silencing mechanism to induce resistance against plant viruses

RNA silencing is a powerful antiviral defense, although viruses have also evolved suppressor proteins to counteract RNA silencing (Tahmasebi and Zangeneh, 2010). Post-transcriptional gene silencing (PTGS) has been developed to induce resistance in plants against viruses. Various constructs have been introduced for enhancing the efficiency of this mechanism (Kavosipour et al., 2012). Hairpins with sense and antisense strands have been used to develop resistance to Barley yellow dwarf virus (BYDV-PAV) in bread wheat (Yasaie et al., 2011). Hairpins of the *b2* gene have also induced resistance to Cucumber mosaic virus (Kavosipour et al., 2012).

RNA interference (RNAi) can be used to degrade the target RNA sequences or prevent their translation through the PTGS process (Kavosipour et al., 2012). Application of RNAi against two subunits of Vacuolar-ATPase enzyme in the insect vector *Peregrinus maidis* showed that targeting vector genes can be a novel strategy for insect control and also for studying the genes involved in controlling

interactions of *Peregrinus maidis* and Maize mosaic virus (Yao et al., 2013). Gene silencing can be used as an effective tool against plant viruses and their vectors.

The role of miRNAs in plants

Plant miRNAs play a role in development, adaptive responses to nutritional stress and biotic and abiotic stresses, metabolism, suppression of plant defenses in order to enable symbiosis with nitrogen-fixing bacteria, nodule formation and symbiosis with rhizo-fungi (Lu et al., 2008; Jones-Rhoades et al., 2006).

miRNAs in antiviral defense

miRNAs have been shown to play an important role in defense against viruses (Lu et al., 2008). In fact, many viruses have the ability to escape the host's RNA silencing pathway. Human adenovirus can stop the synthesis of host miRNAs which would prevent its replication; tissue culture experiments have shown that primate foamy virus type I (PFV-1) can escape RNA silencing mechanism mediated by miR-32 using a silencing suppressor protein known as Tas. miRNA-based defense is not always able to overcome viral attacks (Wang et al., 2007). It has been proposed that plant miRNAs-based RNA silencing plays two main roles in an antiviral defense response: targeting viral RNAs and triggering the biogenesis of siRNA responsible for the antiviral defense.

Antiviral function of endogenous miRNAs has been described in animal systems (Qu and Fang, 2007).

Interaction of viral suppressors and miRNAs in plants

Many viruses encode silencing suppressors which directly interfere with the miRNA pathway. HEN1-dependent methylation of 3'-terminal nucleotides is an important step in the formation of plant miRNAs. Several viral suppressors of RNA silencing (P1/HC-Pro, p21, p19) prevent methylation of miRNAs by HUA ENHANCER1 (HEN1) methyl transferase. The 21 kDa protein p21 of Beet yellows virus, the 19 kDa protein p19 of Tomato bushy stunt virus and P1/HC-Pro silencing suppressor of Turnip mosaic virus all inhibit the miRNA pathway (Yu et al., 2006). Some viral suppressors of RNA silencing inhibit the function of AGO proteins that have a pivotal role in the antiviral RNA silencing (Carbonell and Carrington, 2015). Taken together, viral suppressors of RNA silencing cause major changes in the plant miRNA-mediated gene silencing pathway.

miRNA targets

miRNAs act as molecules to direct the silencing complex toward target mRNAs, and it is necessary to understand the mechanisms by which miRNAs recognize the target RNA and target mRNAs can be predicted for a miRNA sequence (Tanzer et al., 2008). A few interactions between miRNAs and mRNAs have been characterized and confirmed that they are available in 'Tarbase' database. Perfect complementarity between miRNA and its target is not necessary, most miRNA-mRNA pairs form imperfect double strands. miRNA-mRNA double strands are asymmetric, with the bond between 5' end of miRNA and 3' end of the target molecule. The average number of targets per miRNA is low in plants and is often limited only to closely related genes (Tanzer et al., 2008).

Detection and prediction of miRNAs and their target sites

There are numerous methods (e.g. PCR, cloning and sequencing and computer-based predictions) for detecting miRNAs and their targets in various organisms. Most miRNAs have been identified using bioinformatic approaches, homology search against known miRNAs and thermodynamic stability analysis of the stem-loop structures of miRNA precursors.

In closely related species, phylogenetic data can also be used to find conserved sequences in stem-loops. Several software packages including RNAmicro, miralign, miRseeker, miRscan, PicTar and Target Scan are available for detecting miRNAs in genomes of various organisms (Meziere and Enright, 2007). PicTar and Target Scan showed better results for detecting miRNAs in the genome (Thomas et al., 2010).

Trans-acting (ta) siRNAs

miRNAs and trans-acting (ta) siRNAs are generated through different pathways in plants, although both lead to degradation of their target mRNAs. miR173 and miR390 regulate the ta-siRNA precursor processing. The generation of pre-ta-siRNA transcripts is accompanied with the help of miRNAs by RDR6 and Dicer. ta-siRNAs regulate expression of target genes (Allen et al., 2005).

Artificial miRNA

Artificial miRNAs (amiRNAs) are produced using endogenous miRNA precursors in which the mature miRNA region is replaced with the target viral genome (Table 1). This method was first used in animals and then in plants. The precursor can be altered without affecting its normal processing and

Table 1. Plant miRNA precursors and target viral regions for resistance against plant viruses

Plant species	MiRNA backbone	Virus	Target viral region
Arabidopsis thaliana	*Arabidopsis* pre-miR159	TYMV, TUMV	P69, HC-Pro
Nicotiana benthamiana	*Arabidopsis* miR159a, miR167b and miR171a	PPV	P1/HC-Pro
Nicotiana benthamiana	*Arabidopsis* pre-miR171a	CMV	2b
Arabidopsis thaliana	*Arabidopsis* pre-miR159	CMV	3'-UTR
Arabidopsis thaliana, Nicotiana benthamiana	*Arabidopsis* pre-miR159	TuMV	P69
Nicotiana tabacum	*Arabidopsis* miR159a, miR167b, and miR171a	PVY, PVX	HC-Pro, TGBp1/p25 (p25)
Solanum lycopersicum	*Arabidopsis* pre-miR159a	CMV	2a and 2b viral genes, 3'-UTR
Nicotiana benthamiana	*Arabidopsis* pre-miR159a	WSMoV	Conserved motifs of L (replicase) gene
Triticum	Rice miR395	WSMV	Conserved region
Vitis vinifera	*Arabidopsis* pre-miR319a	GFLV	Coat protein (CP)
Nicotiana benthamiana	Cotton pre-miR169a	CLCuBuV	V2
Solanum lycopersicum	*Arabidopsis* premiR319a, Tomato pre-miR319a and pre-miR168a	ToLCV	The middle region of the AV1 (coat protein), the overlapping region of the AV1 and AV2 (pre-coat protein)
Nicotiana benthamiana	*Arabidopsis* pre-miR319a	PVY	CI, NIa, NIb, CP
Zea mays	Maize pre-miR159a	RBSDV	Conserved region
Nicotiana benthamiana	Barley pre-miR171	WDV	Conserved region
Oryza sativa	Rice pre-miR528	RSV, RBSDV	Middle segment, 3' end and 3'-UTR region of the CP gene
Nicotiana benthamiana	*Arabidopsis* pre-miR159a	CBSV, UCBSV	P1, P3, CI, Nib and CP
Nicotiana benthamiana	*Arabidopsis* pre-miR159a	TSWV	N, NSs
Nicotiana benthamiana	Six amiRNAs	PSTVd	Structural domains

TYMV, Turnip yellow mosaic virus (Potyviridae); TuMV, Turnip mosaic virus (Potyviridae); CMV, Cucumber mosaic virus (Bromoviridae); PPV, Plum pox virus (Potyviridae); PVY, Potato virus Y (Potyviridae); PVX, Potato virus X (Alphaflexiviridae); WSMoV, Watermelon silver mottle virus (Bunyaviridae); WSMV, Wheat streak mosaic virus (Potyviridae); GFLV, Grapevine fan leaf virus (Secoviridae); CLCuBuV, Cotton leaf curl Burewala virus (Geminiviridae); WDV, Wheat dwarf virus (Geminiviridae); RSV, Rice stripe virus (unassigned); RBSDV, Rice black streaked dwarf virus (Reoviridae); CBSV, Cassava brown streak virus (Potyviridae); UCBSV, Ugandan cassava brown streak virus (Potyviridae); TSWV, Tomato spotted wilt virus (Bunyaviridae); PSTVd, Potato spindle tuber viroid (Pospiviroidae); ToLCV, Tomato leaf curl virus (Geminiviridae) (Liu et al., 2017).

secondary structure. amiRNAs should not have any sequence complementarity with other parts of the host's genome. Another point is that they should have the minimum hybridization energy with their targets (less than -30 kcal/mole).

A convenient and fast way to design amiRNAs is using the Web miRNA designer service. The 21-nt amiRNA sequence should not have mismatches at nucleotides 10 or 11, as these regions are binding sites of miRNA with its target. Preferably, there should also be no mismatches at 5' end. Some of the precursors including *Arabidopsis* MIR319a, rice MIR528 and *Chlamydomonas* MIR1157 are used to design amiRNAs (Table 1). Constructs with strong promoters (e.g. CaMV 35S) are highly expressed in plants. Overlapping PCR is used for mutagenesis and introduction of the viral sequence into a pRS300 vector harboring miRNA precursor. A 20 bp part of MIR319a is replaced with the 21 bp viral sequence. The pRS300 plasmid is derived from pBluescript. The 21-nt viral sequence is inserted into pRS300 by overlapping PCR method. amiRNA is inserted into the pBI221 vector and then introduced into pCAMBIA 1300 binary vector for cloning and expression of the p35S-miRNA construct. The binary vector is finally transformed into *Agrobacterium tumefaciens* for the production of transgenic plants. Tissue-specific or inducible promoters can also be used to control the expression of amiRNAs. amiRNA-directed degradation of the target is confirmed by RACE-PCR which amplifies degraded segments. Sensitive and resistant plants can be checked using ELISA and PCR methods and symptoms of viral infection (Qu and Fang, 2007; Pérez-Quintero and López, 2010).

amiRNAs-mediated resistance to plant viruses

Plant miRNAs have the potential to trigger antiviral defense, this theory is supported by bioinformatic analyses. Transgenic *Arabidopsis* and tobacco plants with amiRNAs containing sequences from Turnip mosaic virus, Turnip yellow mosaic virus and Cucumber mosaic virus have shown resistance to these viruses. The precursor of grapevine miR166f has been used to produce amiRNAs by replacing 21 nucleotides of Grapevine virus A with 21 nucleotides of the precursor. The viral sequences used in that study were ORF1 (replicase) and ORF5 (RNA silencing suppressor). amiRNAs induced resistance to Grapevine virus A in tobacco (Roumi et al., 2012). amiRNAs could also prevent replication, movement and transmission of the virus. Using viral suppressors of RNA silencing sequences in amiRNAs will therefore enhance plant resistance against viruses (Pérez-Quintero and López, 2010).

Advantages and disadvantages of amiRNAs

Advantages

The amiRNA method can be used to simultaneously silence a group of associated genes or selectively silence a gene in an inducible or tissue-specific manner. amiRNAs have been successfully applied to silence genes in dicotyledons (*Arabidopsis*, tomato and tobacco), monocotyledons (rice), mosses and algae. Precursors from a plant species can be expressed in other plant species and effectively induce gene silencing. amiRNAs have the ability to silence a specific viral sequence. It was shown in a study that the majority of tobacco plants transformed with miR2b showed no abnormal phenotypes; and the plants expressed the highest miR2b indicated no viral symptoms and accumulation of Cucumber mosaic virus. On the contrary, plants with low miR2b levels were highly susceptible. It is speculated that miR2b copies were not sufficient to activate the host's RNA silencing and suppress the viral infection (Qu and Fang, 2007; Pérez-Quintero and López, 2010). Compared to other methods, amiRNA-based silencing shows fewer environmental problems. The size of the transgene is relatively small and the risk of horizontal transfer of viral genes is therefore reduced. Production of new allergens or toxic proteins in transgenic plants is probably less (Pérez-Quintero and López, 2010). amiRNA transgenes are often stable and remain active in the offsprings. amiRNAs have retained their activity up to 6 months and 500 generations in *Chlamydomonas*. Such advantages can make the amiRNAs as the best choice to produce transgenic resistant plants against viruses. amiRNAs can be designed for selectively silencing an allele or a certain form of a gene. The target sequence should be chosen from conserved parts of viral genomes, so that it can induce resistance to various viral strains. miRNA expression level is independent of temperature in *Arabidopsis*, but a number of reports suggest that miR2b expression in tobacco is lower at 15°C than 25°C. The difference between *Arabidopsis* and tobacco may be due to the fact that *Arabidopsis* normally grows at 15°C, whereas this is different for tobacco.

Expression of amiRNAs in plants will probably result in better protection against viral infections (Qu and Fang, 2007; Pérez-Quintero and López, 2010).

Disadvantages

Virus variants with minor differences in the target sequence are difficult to escape this type of silencing mechanism. Viral genomes evolve faster than plant miRNAs and viruses will be able to escape amiRNAs by mutations or deletions in target sequences. This is a barrier to the development of gene therapies for diseases. Point mutagenesis in viral target sequences has been occurred to escape this phenomenon in plant viruses (Plum pox potyvirus and Turnip mosaic virus). amiRNAs from several different sequences of a virus or even from multiple viruses, and preferably using highly conserved sequences might serve as a solution to overcome these problems.

Conclusion

amiRNA is a novel and effective technique to produce transgenic plants. This method can be applied to determine gene function with a very high speed and without the need for large populations or fully sequenced genomes. Advantages of this method include high specificity and lack of recombination with viral genomes (which significantly reduces biosafety risks). Another point is that amiRNA-mediated silencing can be stable at lower temperatures. Research on amiRNAs has also helped the development of other related methods, including ta-siRNA. amiRNAs can also be widely used in plant genetic engineering and provide an efficient mechanism with advantages such as stability, environmental safety and high specificity.

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