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Plant miRNAome and antiviral resistance: a retrospective view and prospective challenges

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Abstract MicroRNAs (miRNAs) are small regulatory RNAs that play a defining role in post-transcriptional gene silencing of eukaryotes by either mRNA cleavage or translational inhibition. Plant miRNAs have been implicated in innumerable growth and developmental processes that extend beyond their ability to respond to biotic and abiotic stresses. Active in an organism's immune defence response, host miRNAs display a propensity to target viral genomes. During viral invasion, these virus-targeting miRNAs can be identified by their altered expression. All the while, pathogenic viruses, as a result of their long-term interaction with plants, have been evolving viral suppressors of RNA silencing (VSRs), as well as viral-encoded miRNAs as a counter-defence strategy. However, the gene silencing attribute of miRNAs has been ingeniously manipulated to down-regulate the expression of any gene of interest, including VSRs, in artificial miRNA (amiRNA)-based transgenics. Since we currently have a better understanding of the intricacies of miRNA-mediated gene regulation in plant–virus interactions, the majority of miRNAs manipulated to confer antiviral resistance to date are in plants. This review will share the insights gained from the studies of plant-virus combat and from the endeavour to manipulate miRNAs, including prospective challenges in the context of the evolutionary dynamics of the viral genome. Next generation sequencing technologies and bioinformatics analysis will further delineate the molecular details of host–virus interactions. The need for appropriate environmental risk assessment principles specific to amiRNA-based virus resistance is also discussed.

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Abbreviations

amiRNA	Artificial miRNA
DCL1	Dicer-like 1
ERA	Environmental risk assessment
EST	Expressed sequence tag
hpRNA	Hairpin RNA
ihpRNA	Intron-spliced hairpin RNA
miRNA	MicroRNA
NBS–LRR	Nucleotide binding site–leucine-rich repeat
NGS	Next generation sequencing
ncRNAs	Non-coding RNAs
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
PTGS	Post-transcriptional gene silencing
RNAi	RNA interference
siRNA	Small interfering RNA
SMRT	Single molecule real time
tasiRNA	Transacting siRNA
VSRs	Viral suppressors of RNA silencing
VRTP	Virus resistant transgenic plants

Introduction

Micro-RNAs (miRNAs) are a class of small non-coding RNAs (ncRNAs) that act as ultimate effector molecules of

post-transcriptional gene regulation in eukaryotes. Plant miRNAs discovered at the beginning revealed that they were conserved across the species and were involved in regulating the expression of transcriptional factors (TFs) or genes of the hormone biosynthesis pathway, thereby controlling the plant's growth and development [1]. The indispensable role of miRNAs at various developmental stages was conclusively demonstrated with the advent of miRNA biogenesis mutants [2–4]. The widespread impact of miRNA activities includes, but is not limited to, embryogenesis [5], flower development, leaf morphogenesis, patterning [6], transitional phasing [7, 8], anther development and reproduction [9, 10]. miRNAs have also been implicated in the plant's response to biotic [11] and abiotic stresses [12, 13]. Spatial and temporal expression of miRNAs, followed by subsequent regulation of target mRNAs, forms the basis of the ncRNA-based gene regulatory networks in plants. The insights gained about this gene regulatory scheme, as well as about the small RNAs landscape (sRNAome), during host–virus interactions have paved the way for directed manipulation of the miRNA pathway such that antiviral resistance in plants could be conferred.

Previously, several strategies of plant genetic modification including the expression of viral coat proteins, replicase proteins, or nucleoproteins had been employed in order to confer resistance against phytopathogenic viruses [14–18]. Furthermore, prior to any comprehension of their molecular basis, RNA silencing phenomena such as antisense suppression and co-suppression were acknowledged to function effectively in plants [19]. In later studies, small interfering RNA (siRNA) was delineated as effector of RNA-mediated gene silencing in a sequence-dependent manner [20–22]. A recent and potent addition to the toolbox of post-transcriptional gene silencing (PTGS) is artificial miRNAs (amiRNAs). Because they can exploit the endogenous microRNA (miRNA) pathway to engineer antiviral resistance, amiRNAs are certainly proving to be valuable tools.

This review will highlight the role that miRNAs play in a plant's defence system, the counter-defence mechanism toward viral pathogens, and the potential applications of miRNA-mediated viral gene suppression. Also explored in this review are: future challenges, likely attributable to the molecular dynamics of viral genome evolution; the advent of techniques such as next generation sequencing (NGS); and lastly, the necessity of specific knowledge and of employing specific environmental risk assessment (ERA) criteria for amiRNA-based transgenics.

miRNA biogenesis

miRNAs are 21–24 nt long, regulatory small RNAs (sRNAs) with biogenesis occurring inside the subnuclear location called as D-bodies [23] (Fig. 1). miRNA biogenesis is initiated from long single-stranded RNAs, called primary miRNA transcripts (pri-miRNAs). pri-miRNAs are characterized as imperfect stem-loop structures which are generated by the activity of RNA Pol II [24]. The conversion of pri-miRNAs, through precursor miRNAs (pre-miRNAs), to a functional miRNA:miRNA* duplex is coordinated by the activity of many protein families including RNAse III, Dicer-like-1 (DCL-1), etc. [25]. The activity of DCL-1 also requires the participation of DAWDLE in recruiting DCL-1 to pri-miRNA [26]. Plant pre-miRNAs, which are relatively long (90–140 bp) compared to the pre-miRNAs of animal origin (60–70 bp long), are further diced into smaller, double stranded mature miRNA (miRNA:miRNA*) inside the nucleus and transported to the cytoplasm by EXPORTIN-5 [27]. In this process, DCL-1 interacts with dsRNA binding proteins like HYL1 (hyponastic leaves1), and the zinc finger protein SE (SERRATE) inside the nucleus to produce mature miRNA [28]. The miRNA is methylated and polyuridyated by HEN 1 (HUA Enhancer 1) which protects it from degradation [29]. The mature miRNA is then exported out of the nucleus by an EXPORTIN orthologue in plants called HST 1 (HASTY 1) [30, 31]. Resultant 21 nt long miRNAs are recruited to the RNA-induced silencing complex (RISC), which either cleaves the complementary mRNA sequence [27, 32], or represses its translation. [33]. The latter mechanism, found predominantly in animal systems, has been recognized to function in the endoplasmic reticulum of plants as well [33, 34]. The effector miRNA in the silencing complex is primarily acquired from one strand of the miRNA duplex (miRNA:miRNA*), whereas the other strand, called the passenger strand (miRNA*), is degraded. Emerging evidence does suggest, however, that the passenger strand (miRNA*) has an active biological role in the sRNA background of plants [35, 36].

Plant miRNAs and the adaptive response to viral invasion

Although the discovery of miRNA dates back to 1993 [37], it was not until a decade ago that miRNAs were implicated in the host's defence mechanism [38]. Subsequently, miRNAs of animal origin were shown to play a role in the suppression of invading viruses [39]. In plants, miR393 was the first host-derived sRNA recognized to function in antibacterial resistance by modulating the auxin signalling pathway [11]. Plant-derived miRNAs were later reported to be associated with the repression of Plum pox virus (PPV) replication in vivo [40]. Several studies available on miRNA-mediated post-transcriptional regulation in response to viral infection have been documented [36, 41, 42]. From

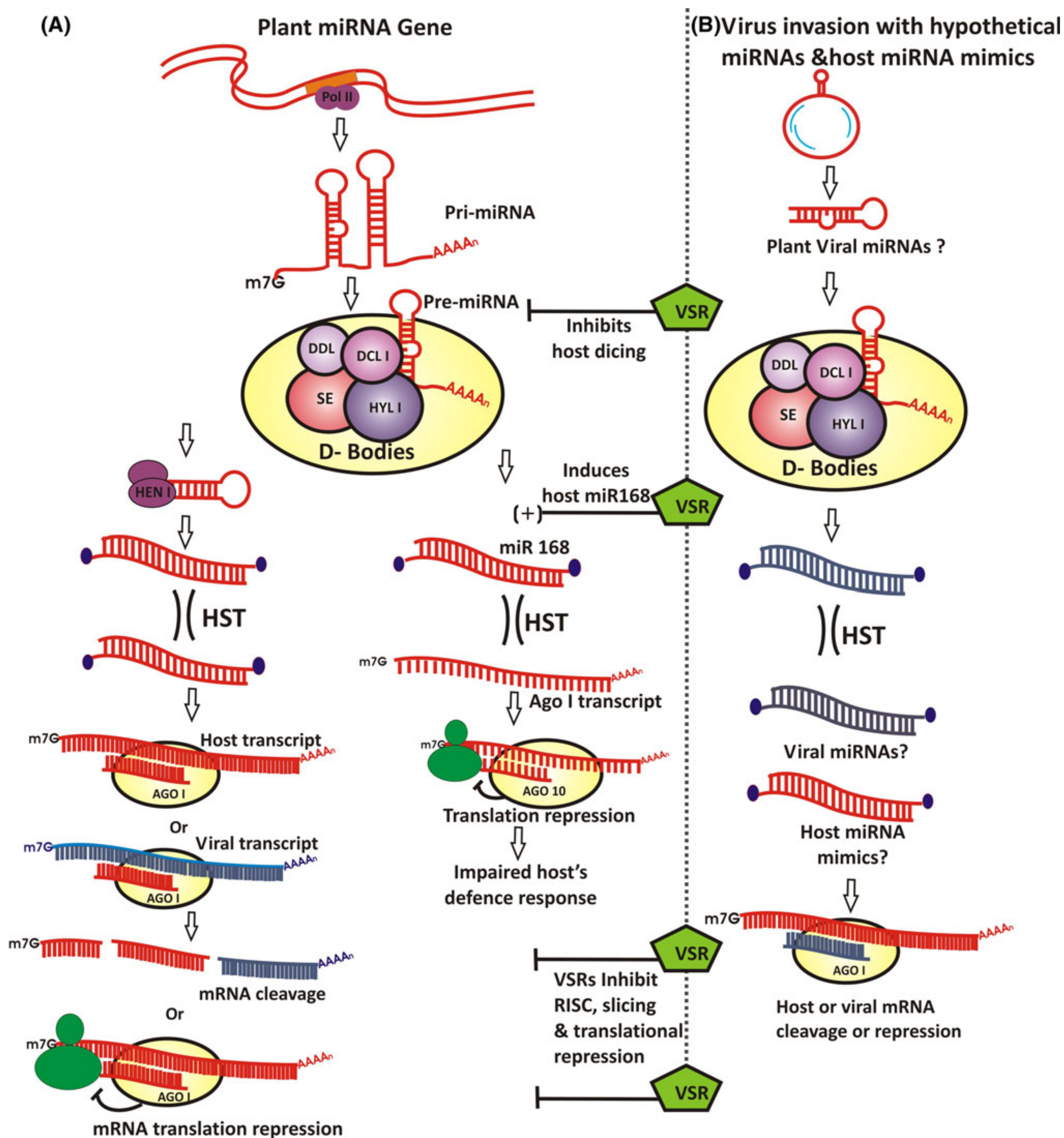

Fig. 1 Overview of plant–virus interface with special reference to the miRNAome. **a** Plant genome encodes miRNAs that target viral transcripts as part of the antiviral response. Viral invasion alters the miRNA landscape of the host by expressing VSRs that inhibit dicing, RISC assembly, slicing, and the translational inhibition activity of the host miRNA mechanism. VSR (p19 of Cymbidium ringspot virus) has been shown to induce the expression of host miRNA 168 leading to the downregulation of the AGO-I protein that is involved in host's defence mechanism. **b** Even though plant virus-derived miRNAs have not yet been documented, this figure illustrates hypothetical viral strategies, including miRNA-mediated repression of the host and viral transcripts. Presented here, as well, is the hypothesis that animal infecting viruses, in general, will adopt the expression of host miRNA mimics in order to shift combat in the pathogen's favour. *Pol II* RNA polymerase-II, *Pri-miRNA* primary miRNA transcript, *DCL* Dicer-like, *DDL* DAWDLE, *SR* SERRATE, *HYL* hyponastic leaves, *HEN* HUA enhancer, *HST* HASTY, *VSR* viral suppressor of RNA silencing, *AGO* ARGONAUTE

these studies, microarray analysis of tomato plants agro-infected with Tomato leaf curl New Delhi virus (ToLCNDV) detected the deregulation of conserved miRNA families including miR319 and miR172 [41]; whereas with another Begomovirus infection, miRNAs involved in plant developmental processes were found to be up-regulated, leading to the suppression of corresponding endogenous targets [42]. A comparative analysis of the sRNA landscape of the host, upon infection with the Rice dwarf virus (RDV) and the Rice stripe virus (RSV), revealed that RSV infection induced expression of novel miRNAs in a phased manner. It was also documented here that the enhanced expression of some miRNAs* (passenger miRNA strands) was concomitant with small changes in the expression levels of corresponding miRNAs. Furthermore, in contrast to infection by a solitary virus, co-infection of *Nicotiana benthamiana* with Potato virus X, Potato virus Y, and the PPV resulted in an altered host miRNA expression profile. Thus, the differential modulation of host sRNA metabolism can be observed under the condition of multiple virus infection [36]. Notwithstanding the plethora of plant miRNA regulatory networks that are operational in plants, it is plausible to deduce a common pattern of miRNA regulation due to viral infection. Uncovering these patterns could prove beneficial to the development of biomarkers for the diseased state or towards imparting plant resistance through antiviral strategies. It has also been suggested that miRNA passenger strands (miRNAs*), previously considered degradation products with little role in vivo, are involved in the antiviral defence mechanism of plants [41]. The conserved and abundantly expressed plant miRNA families (miR156, miR159, miR319, miR172, etc.), in general, merit discussion, as they are thought to have a repressive role toward viral invasion. Computational [43] and microarray-based experiments have provided evidence that conserved miRNAs generally demonstrate greater antagonism toward viral genomes [41].

The assumption that those miRNAs, which were able to confer defence against viral invasion, would have survived evolutionary selection and became conserved, provides an explanation for the abundance of conserved miRNAs in the plant small RNAome. It also follows from this assumption that any supplementary functions that miRNAs exhibit would have most likely been acquired by them at later evolutionary stages. The occurrence of ORFs encoding viral suppressors of RNA silencing (VSRs) in the genomes of plant viruses, with their primary function of debilitating the host's sRNA metabolism, stands as evidence in support of this latter hypothesis. The hypothesis is further supported by the duplication and divergence mechanism of miRNA evolution. The mechanism reveals that miRNA families that are conserved across species exhibit copy number variation, followed by qualitative sequence differentiation, which together, are thought to be leading to the evolution of miRNAs with the emergence of novel functions [44].

It has also been hypothesized that even plant miRNAs that are relatively less abundant could conceivably cater to the host's defence mechanisms during specific host–virus interactions. Of late, miRNAs have also been implicated in the regulation of plant innate immune responses by modulating nucleotide binding site–leucine-rich repeat (NBS–LRR) genes in legumes [45] and solanaceae [46, 47]. In NBS–LRR-mediated non-specific immunity, when there is absence of infection by a pathogen, only a few miRNAs control the cascade of defence proteins. Conversely, defence proteins under miRNA control are triggered instantly, upon viral invasion, as VSRs derepress miRNA-based control of defence proteins. Thus, it appears that miRNA-mediated modulation of plant defence mechanisms functions on the principle of cellular economy. Another perspective on the presence of host-derived miRNAs is that these viral responsive miRNAs, by not targeting all the viral ORFs, might be enabling co-existence of viruses inside the host, and thereby allowing the establishment of a persistent infection [48]. This perspective is plausible considering our deprived understanding of the sRNAome landscape of plants in general, and in particular, of the regulation of virus–plant interactions.

In silico miRNA target predictions and viral genomes

The immense number of small ncRNAs involved in plant gene regulatory mechanisms combined with the lack of high throughput biological methodologies to assess global miRNA expression patterns has led to a reliance on computational approaches. As our understanding of the plant–virus miRNAome interface is far from complete; miRNA and target prediction algorithms are still evolving. Various miRNA target prediction algorithms have been developed, which are based not only on sequence complementarity [49], but also on thermodynamic properties which evaluate energetically favourable binding sites [50–52]. A few algorithms also operate on machine learning techniques based on the features of, and selected patterns from actual miRNA–target interactions [53–56]. Furthermore, if the assumption is accepted that abundant non-conserved RNAs with unresolved target sequences serve as prospective, in vivo antiviral reservoir molecules, then bioinformatics approaches not only become an interesting area for research but will also serve as powerful application tools.

Investigations into the antiviral attributes of miRNAs derived from six plant species uncovered that plant miRNAs preferentially target genomes of phytopathogenic viruses, as compared to negative controls, which included

randomly generated miRNAs or genomes of animal viruses [43]. Likewise, research on several tomato-derived passenger miRNA strands (miRNA*) has unearthed the propensity of miRNAs* for binding to the ToLCNDV-associated genomes. Their use in these previous investigations corroborates that computational approaches are robust for delineating the intricacies of miRNA-based gene regulation. Thus, computationally predicted miRNA effectors against viral genomes cannot be dismissed completely on the grounds that they arose out coincidental, fortuitous pairings. It has been established through genetically modified plant miRNAs (amiRNAs) that sequence complementarity between altered miRNA sequences (within the endogenous precursor backbone) and the viral genome successfully commences the down-regulation of target viral transcripts [57–60]. To summarize, predicted miRNA target sites have shown that they have the potential to develop amiRNA-based antiviral defence in plants.

miRNA and viral counter defences

RNA silencing is an evolutionarily conserved pathway. In response, viruses have evolved genome-encoded VSRs and miRNAs as counter-strike measures to incapacitate the defence mechanism of plants [60–62] (Fig. 1). Suppressors of RNA silencing such as 2b of Cucumber mosaic virus (CMV), P1/HC-Pro of the Potyvirus, p19 of Tombusviruses, p38 encoded by the Turnip crinkle virus, ORFs AC-4 and AC-2 encoded by Geminiviruses, etc. have been characterized in several families of plant-infecting viruses [63–67]. VSRs have been reported to display various modes of action including binding to dsRNA, inhibition of DCL dicing activity [68, 69], binding to siRNA, sequestration [70–72], inhibition of silencing machinery [73], preventing spreading of the gene silencing signal [74, 75], modification of sRNA methylation [76–78], mimicking host protein motifs involvement with AGO binding [79, 80], inhibition of antiviral silencing signal amplification [81], and varying epigenetic modifications on viral [67, 82] or host genomes [83].

The role of VSRs in debilitating host miRNA pathways also deserves its own discussion. The molecular basis behind the manifestation of viral symptoms lies in the ability of VSRs to interfere with host miRNA biogenesis, ultimately affecting host mRNA turnover to the advantage of invading pathogens [84–87]. A p19 VSR of Cymbidium ring spot virus, for instance, induces host-derived conserved miRNA 168 that is involved in restraining AGO-1 accumulation. As AGO-1 accumulation is crucial for the antiviral function of RISC, host miRNA modulations, under the influence of viral infection, lead invariably to an impaired host antiviral response [88, 89].

Interestingly, VSR 2b of CMV has been shown to exhibit miRNA modulating activity and symptom induction, independently of one another, leading to the conclusion that the RNA suppressor domain acts discretely from the host miRNA inhibitory domain [90]. A report on two unrelated VSRs (Potyvirus HC-Pro and Carmovirus p38) revealed viral activities that were consistent with the notion of distinct domains. In addition, it is known that host TFs are involved in HC-Pro-mediated morphological anomalies but not in their miRNA inhibitory role [91]. Furthermore, the differential effect of VSRs on siRNA and miRNA AGO-1 loading proposes the presence of two different pools of ARGONAUTE proteins in vivo [92]. To summarize, because the plant's antiviral defence and endogenous gene regulatory networks share common protein machinery, which would otherwise be involved in maintaining normal cellular processes, leading to the manifestation of disease symptoms.

Animal viruses have been found to encode miRNAs that effectively regulate viral gene expression and amend the host's sRNA metabolism [93]. The earliest existence of viral genome encoded miRNAs was detected in Epstein–Barr virus (EBV)-infected B cells [94]. Since then, more than 200 viral encoded miRNAs have been identified among metazoan-infecting viruses, primarily in Herpesviruses, Polyomaviruses, and Retroviruses [95, 96]. Besides controlling the expression of the host transcriptome, viral miRNAs are involved in regulating their own transcripts, which is essential for the transition from latent to lytic gene expression [97, 98]. However, scrutiny of analogous miRNAs in the genomes of plant-infecting viruses has provided little evidence towards the existence of virus-derived miRNAs. An explanation for why metazoan virus-encoded miRNAs exist, while plant analogues have yet to be uncovered, may lie within the mode of action of animal-infecting viruses. In general, metazoan miRNAs repress mRNA translation, a process that entails time, makes miRNAs a potent mode of viral gene regulation, through which viruses then display characteristic features such as latency or persistent infection. Access to nuclear RNAse, like Drosha, involved in miRNA biogenesis, is another major factor that may explain the prevalence of viral-encoded miRNAs in metazoan DNA viruses including Herpesviruses and Retroviruses. Findings by Kincaid et al. [96] with supporting evidence from Klase et al. [99] emphasize that the presence of retroviral-encoded miRNAs in animals encourages the role of viral-encoded small ncRNAs in determining the outcome and disease course.

One plausible reason for the general absence of plant viral encoded miRNAs may be that viruses that infect plants are RNA viruses, and RNA viruses are not known to enter the plant cell nucleus. Nevertheless, detection of both viral strands of Turnip mosaic virus (TuMV) within the nucleus stands to show that RNA viruses, in fact, do enter the nucleus [100]. Correspondingly, a probe of the genome

of TuMV for the loci responsible for miRNA generation, using miRNA prediction algorithms as well as experimental verification, uncovered two novel miRNAs, namely, TuMV-mir-S1 and TuMV-mir-S2. It was further deduced that these viral miRNAs target the abscissic acid-regulated gene HVA22D in Arabidopsis, thus generating molecular cross-talk between biotic and abiotic stress pathways in plants [100].

Robust sRNA profiling systems and NGS technologies on the molecular level are expected to decode the complete repertoire of sRNAs that are involved in the intricacies of host defence and viral counter-defence measures. It is likely that in the plant–virus interface, widespread incidence and dependence on VSR proteins as the primary viral counter-defence mechanism, which would make the role of viral encoded miRNAs redundant.

Plant miRNAome engineering for antiviral resistance

Engineering of amiRNA for antiviral resistance involves the use of host-derived endogenous precursor miRNA, as a structural backbone, to replace the original 21 nt long miRNA sequence with the region complementary to the target viral genome. Prior to its use for promoting antiviral resistance, altered miRNAs backbones, termed amiRNAs, had been successfully exploited for the targeted down-regulation of endogenous genes [101, 102]. Encouraged by the premise that alteration of the 21 nt long effector nucleotide sequence of miRNA gene does not modify its biogenesis, researchers decided to deploy endogenous host-derived miRNA backbones as an effective RNA silencing tool in planta (Table 1). With that strategy, it became necessary to confirm that secondary structural features such as complementary mismatches or bulges remained intact within the precursor miRNA so that miRNA processing in vivo would remain unaltered [103, 104].

The selection of amiRNA sequences is guided by specificity and effectiveness of silencing. The sequence chosen for the 21 nt long amiRNA should be unique so that only specific viral transcripts of interest are down-regulated. To preclude any amiRNA induced off-target effects, it would, nonetheless, be necessary to scan the host plant transcriptome for unintended matches. The complete genome sequence of the host species, wherever available, or ESTs in the transcriptome database, could play a crucial role in the scanning process [59]. In other crops, however, off-target effects would have to be analyzed as a post-transformation exercise. Effectiveness of amiRNA is measured by parameters such as maximum sequence complementarity with the target gene, thermodynamically minimum hybridization energy between the amiRNA and target RNA, etc. [105]. In addition, nucleotide incorporation features (A or U at position 10 and U at the first position) on the effector miRNA sequence are advantageous because these consensus nucleotides are adequately represented in the biologically active miRNAs [102, 106, 107]. Similarly, avoidance of mismatches at amiRNA nucleotide positions 10 or 11 (prevents proper cleavage of target mRNA) and a provision for instability at the 5′ end of amiRNA (enhance the likelihood for RISC incorporation) are some characteristics to be considered for the effective design of antiviral amiRNA.

The accessibility of target viral RNA for amiRNA silencing is an equally important consideration. Whereas in silico secondary structure prediction of target viral transcripts is possible [108, 109], it has been insufficient to mimic in vivo conditions. An ingenious experimental procedure allows for the determination of accessible spots on viral RNA by comparing the viral-derived siRNAs from wild-type Arabidopsis with sRNAs derived from DCL mutants. The target viral transcript is thus assessed for DCL susceptibility and the vulnerable region is identified around which antiviral amiRNAs can be designed [110]. Further, homologous in vivo recombinant amiRNA vectors have been successfully generated to silence plant endogenous genes, which reduces the time and cost involved in efficient amiRNA plasmid construction [111].

Deliberations on amiRNA-based antiviral resistance

amiRNA-based viral resistance, expression of viral genome-derived sequences to confer resistance against infecting viruses is fraught with various risk factors including possible recombination between the transgene and non-target viruses in field conditions and conceivable synergism between the transgene and unrelated viruses. In protein expression strategies, the risks may vary from production of toxic proteins or allergens, transencapsidation, to transmission of viral proteins by incompatible insect vectors. In contrast, amiRNA-based virus resistance does not carry potential environmental risks like toxicity, allergen production, transencapsidation, etc.

Studies on directed viral genome recombination have revealed that it is selectively detrimental for both the viruses involved to undergo recombination [112]. To date, no recombination events have ever been documented involving transgene-derived sequences and natural viruses in field conditions. By contrast, in an amiRNA-based situation, because the effector molecule of RNA silencing is a 21 nt long, viruses exhibit the likelihood of evolving complementary, vulnerable genomic regions through mutation. Equipped with such regions, viruses could thus evade the amiRNA silencing mechanism. Given the plasticity of RNA viral genomes, and of other viruses where

Table 1 Plant miRNAome engineering for antiviral resistance

Plant species	miRNA backbone	Virus and family	Target viral region/gene	Features
Arabidopsis thaliana	*Arabidopsis thaliana* pre-miR159	*Turnip yellow mosaic virus* *Turnip mosaic virus* (Potyviridae)	P69 HC-Pro, coat protein	Dimeric pre-amiRNAs targeting P69 and HC-pro [48]
Nicotiana benthamiana	*Arabidopsis thaliana* pre-miR171a	*Cucumber mosaic virus* (Bromoviridae)	2b	Efficacy of amiRNA over siRNA-based viral gene silencing [49]
Arabidopsis thaliana	*Arabidopsis thaliana* pre-miR159	*Cucumber mosaic virus* (Bromoviridae)	3′UTR	[110]
Arabidopsis thaliana	*Arabidopsis thaliana* pre-miR159	*Turnip yellow mosaic virus* (Potyviridae)	P69	Stability of amiRNA-mediated viral resistance and mutations on viral genome assessed [114]
Arabidopsis thaliana	*Arabidopsis thaliana* pre-miR159	*Turnip yellow mosaic virus* (*Potyviridae*)	P69	Chimeric [114]
Nicotiana benthamiana	Arabidopsis thaliana pre-miR159	*Turnip yellow mosaic Virus* P69 Chimeric (Potyviridae)	P69	[114]
Nicotiana benthamiana	*Arabidopsis thaliana* miR159a, miR167b and miR171a	*Potato virus Y* (Potyviridae) *Potato virus X* (Alphaflexiviridae)	HC-Pro TGBp1/p25 (p25)	Multiple virus resistance through expression of dimeric amiRNA precursor [131]
Tomato	*Arabidopsis thaliana* pre-miR159a	*Cucumber mosaic virus* (Bromoviridae)	2a and 2b viral genes 3′ UTR	Resistance against non-target viruses like TMV and TyLCV Cell autonomous gene silencing [119]
Nicotiana benthamiana	*Arabidopsis thaliana* pre-miRNA159a	*Watermelon silver mottle virus* (Bunyaviridae)	Conserved motifs of replicase	Triple amiRNA constructs conferring complete resistance [132]
Wheat	Rice miR395	*Wheat streak mosaic virus* (Potyviridae)	Conserved region	Polycistronic amiRNA precursor (FanGuard-FGmiR395) [127]
Grapevine	*Arabidopsis thaliana* pre-miRNA 319a	*Grapevine fan leaf virus* (Secoviridae)	Coat protein	[51]

recombination and mutational genomic rearrangements are not uncommon, such mutational events are plausible.

The rapidity of viral genome evolution against the inhibitory actions of host-derived miRNAs has been observed in PPV chimeras containing genomic miRNA target sites [113]. Likewise, experiments assessing the evolutionary stability of amiRNA-mediated resistance in the TuMV revealed that these viruses have circumvented the RNA silencing process by rapidly accumulating mutations in the target genomic regions [114]. Population dynamics studies of the TuMV, which is under the influence of amiRNA expressing plants, revealed the emergence of escape alleles characterized by at least one nucleotide substitution within the target genomic region. Moreover, mutations accumulated at a faster rate when the viral population was exposed to suboptimal concentrations of amiRNAs [115].

Developments in the field of deep sequencing have enabled the study of the virus population while it is under the influence of sRNA-based resistant plants. One study proposes that the evolution of virus genomes, vis-à-vis the plants that expresses resistance, involves a complex dynamics of mutation, selection, and drift [116]. Resistant-breaking strains or escape variants could accelerate the development of virulent viral strains such that it would become too cumbersome to counteract them through available modes of virus control. Hence, resistance management strategies need to be prioritized ahead of the cultivation of amiRNA-based transgenics. A type of situation, similar to that described above, is anticipated with the increased use of virus-resistant transgenics based on hairpin (hpRNA)-generated siRNAs. One key difference with this type of transgenics is that the pool of in vivo diced siRNAs generated from the hpRNA arm targets relatively long regions of the viral genome, imposing little selective pressure on any particular viral target region and thus, making the chance for the rapid development of resistant-breaking strains moderately low.

The conserved genomic region of the virus is considered the appropriate target for amiRNAs. This is the case because amiRNA induced mutational changes in conserved regions would be selected against within the natural viral population because of their detrimental effects. It is important to acknowledge at this point that a computational analysis of tomato-derived miRNAs against the genome of an infecting virus revealed that the principle of co-operative binding (where an ORF of the virus genome is under the target of multiple host miRNAs) was in effect [117]. Furthermore, as amiRNA silencing processes function in a cell autonomous manner, silencing signals were not transmitted systemically [118]. With the above concerns in mind, and in order to combine the durability of hpRNA generated siRNAs along with its tissue specificity, and temperature insensitivity of amiRNA strategies, it is advisable that multiple amiRNAs are expressed to target specific viral genomic regions.

Due to their pervasiveness as they are encoded by the majority of viruses-VSRs that are disruptive to miRNA-mediated gene repression, in particular. In the presence of suppressors, the miRNA passenger strand (miRNA*)—an unstable intermediate—accumulates at a high enough concentration to inhibit the miRNA-mediated cleavage of target mRNAs [84]. Numerous studies have confirmed that viral genome-encoded suppressors such as the p122 subunit of the Tobacco mosaic virus replicase, p19 of Tombusvirus, etc. preferentially sequester double stranded 21 nt long miRNAs and hamper its recruitment to the RNA-induced silencing complex (RISC) [72, 87]. To enhance the effectiveness of amiRNA-based antiviral resistance, an environment abundant with antiviral effector miRNAs and absence of VSRs are considered conducive. It is therefore appropriate to develop amiRNAs that target VSRs themselves.

siRNAs are another class of extensively employed regulators that confer antiviral resistance in plants. siRNAs originate from moderately long ssRNA which form perfectly complementary dsRNA. Conversely, miRNAs originate from ssRNAs that display imperfect sequence complementarity on their arms. Notwithstanding the differences in their biogenesis, both sRNAs are involved in the downregulation of target transcript expression. miRNAs always act in trans by targeting RNA in a sequence-dependent manner and demonstrate little activity in cis. On the other hand, siRNAs act both in cis and trans, thereby repressing any target RNA that displays substantial sequence complementarity.

The divergence between the sRNAs further extends to their mode of action and signal amplification strategies. Grafting experiments revealed that amiRNAs lack systemic movement as amiRNA expressing rootstocks could not prevent the wild-type scion tissues from being afflicted with disease, which indicates that amiRNAs remain localized and act in a cell autonomous response [119]. On the other hand, siRNAs, cognate to the viral genome, exhibit long-distance movement through the phloem to get to uninfected tissues and confer resistance to distant cells [99]. These experiments have shed light on the complexities behind sRNA-mediated gene silencing. Unlike siRNA-mediated viral gene repression, which functions only after the onset of viral infection, cells previously exposed to antiviral miRNAs are primed for imminent viral infection.

Another advantageous feature called transitivity—the ability to generate secondary siRNAs from proximal primary siRNA targets—had previously been considered to be active only in siRNA-mediated RNA cleavage. Transitivity has since been elucidated and was found to be instigated, as well, by 22 nt long miRNAs, acting in concert with trans-

acting siRNA (tasiRNAs) in Arabidopsis. Furthermore, because miRNAs can act against the target RNA despite nucleotide mismatches, it can serve as a tool to modulate multiple targets, and in doing so, expand the spectrum of its action. Despite the fact that siRNAs and miRNAs share overlapping machinery, miRNAs have been able to widen their target range due to the presence of complementary mismatches and function as molecular support to siRNAs in defence against invading pathogens. The result of all the above findings is an expanded view of the potential target range of amiRNAs [120].

Prospective challenges

Although our understanding of the plant-virus miRNAome interface continues to improve, pertinent questions remain yet unanswered. Do virus responsive miRNAs have supplementary cellular functions? Do complementary mismatches in the miRNA structure necessarily translate into an expanded spectrum of action? The versatility of miRNA functions in the cellular milieu leads to the proposition that virus responsive miRNAs do have auxiliary functions. In this scenario where virus responsive miRNAs have additional roles, what are the in vivo consequences of constitutively expressed virus responsive miRNAs? Another query: Whether plant virus derived miRNAs do exist, and if so, whether those miRNAs act as host miRNA mimics, or what is their mode of action?

Understanding the virus–plant miRNAome is valuable to understanding pathogen–host interactions that could then pave the way for engineering miRNA for targeted antiviral resistance. In the miRNAome dissection studies, the target mimicry approach, one of the most reliable and functional genomics tools used to knockdown the activity of miRNAs, involves the expression of an uncleavable target transcript that preferentially binds and sequesters the cognate miRNA, restricting it from performing its original cellular activity [121]. In addition, miRNA arrest—miRNA misprocessing leading to the production of truncated miRNA—is an alternative strategy that has been recently employed in miRNA functional genomics studies as well as in potential gene therapy applications [122]. Due to its high throughput abilities, specificity, and sensitivity, NGS has proven to be an extremely valuable tool for ascertaining miRNA expression profiles, and for discovering miRNA isoforms or biomarkers for diseased states or developmental stages [46, 123]. Single molecule real time (SMRT) sequencing platforms, a variant of NGS, are expensive to run, and thus are not being widely utilized; however, they seem to offer the potential for greatly encouraging miRNA studies in the future [124].

The enormous amounts of data predicted to be generated from NGS studies will require use of robust computational platforms, based on heuristic search algorithms and parallel computing techniques, for completing downstream analysis. Plant miRNA target prediction algorithms are currently moving towards machine learning approaches which are more suitable for global transcriptome analysis because they do not designate sequence complementarity as the sole criterion for successful miRNA: target interactions. Instead, these recent algorithms consider relevant critical features such as the secondary structure of target RNA, miRNA-mediated translational inhibition, target site accessibility, etc. [125, 126]. In addition, plant miRNAome is characterized by far-reaching gene duplications that are discretely located as divergent family members with varying copy numbers throughout the genome. Better understanding of the evolutionary dynamics of miRNAs across various species, in the context of virus–host interactions, would provide a more comprehensive view of the miRNAome at the plant–virus interface.

Despite the fact that some of our understanding of the plant miRNAome and virus resistance is less than complete, amiRNAs have been successfully deployed to combat virus infections, not only in model plants, but also in agriculturally important crops [60, 119, 127]. But the successful field deployment of amiRNA-based virus-resistant transgenic plants also necessitates the practice of sound predictive ERA principles. One of the main ERA concerns surrounding RNAi-based transgenics, in general, and amiRNA-based virus resistant transgenic plants, in particular is the likelihood of off-target effects [128, 129]. Of equal concern is the likelihood of non-target effects of amiRNAs against the transcriptome of organisms which happen to be in the vicinity of amiRNA-expressing plants. In order to avoid potential non-target and off-target effects, gaps in knowledge regarding exposure routes and the environmental fate of amiRNAs need to be thoroughly assessed. Furthermore, molecular cross-talk studies, post-amiRNA expression, will also be essential for deciphering any inadvertent transcriptome changes in planta. Another potentially serious ERA scenario to keep in mind is that through the constitutive expression of amiRNAs, host RNAi machinery could experience unintended overload, adversely impacting the general defence mechanisms of the host.

The cross-kingdom presence and activity of plant sRNAs [130] against the mammalian system warrants meticulous food and feed safety measures to be able to detect the smaller RNA fragments such as amiRNA, in a sensitive, yet robust manner. It should be noted, however, that consumption of virally derived nucleic acids has not, to date, created any serious food/feed safety concerns. There exists, in fact, a long history of the safe consumption of virus-infected economic plant parts. Being able to detect

sRNAs (amiRNAs and siRNAs) and then to distinguish them from naturally occurring sRNAs that are generated as a result of host innate antiviral defence mechanisms, remains a challenging task for amiRNA-based VRTPs. Another feature of transgenics, the persistence of sRNAs, especially amiRNA hairpin structures in vivo will need to be measured and their environmental stability assessed. The level of persistence of amiRNAs in the receiving environment can be measured by estimating the degradation time to 50 % loss (DT_{50}) for hairpin RNAs. In addition to the above consideration, other conventional ERA features like altered invasiveness or weediness and any adverse impact on cultivation practices arising due to amiRNA-based transgenics warrant detailed investigation.

Conclusions

With the advent of NGS platforms and the rapid discovery of miRNAs in various plant species, the potential of miRNA-ome-based gene repression phenomenon as a valuable genetic engineering instrument is being realized. The knowledge gained from current techniques confirms that viral resistance is in itself, an important area of research, especially with regard to economically important crops. Besides helping to realize virus resistance in economically important crops, the technique also facilitates reverse genetics, with wide ramifications for investigations involving virus–host interactions. The revelation that amiRNAs are highly flexible along with an expanded activity horizon means that miRNAs could be deployed effectively against conserved nucleotide sequences of viral genomes to obtain broad spectrum resistance. Tissue-specific gene knock-out, temperature insensitivity, and the prospective stacking of multiple amiRNAs against distinct viruses or multiple genomic regions of the same virus are all features that contribute to the expediency of miRNA-based viral gene silencing. Nevertheless, elucidation of the molecular mechanisms underlying host antiviral immunity with respect to VSRs and miRNAs will allow us to more thoroughly harvest the benefits derived from the amiRNA-based gene silencing mechanism.

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