Non-Coding RNAs and Plant Antiviral-Defense

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RNA technologies provide us not only with new sources of resistance but also with powerful tools to manage plant defense responses. With regards to antiviral resistance, RNAi-based approaches have long been used in the form of transgene-induced RNA silencing to target the degradation of diverse viral RNAs or to inactivate virus susceptibility genes in various crops.

Keywords: dsRNA ; siRNA ; miRNA ; lncRNA ; amiRNA ; tasiRNA ; RNAi ; CRISPR-Cas

1. Introduction

Due to climate change and alterations in cropping systems, plant viral disease outbreaks will likely have increasingly negative impacts on food production, which will bring new challenges to current agricultural practices worldwide. To advance virus disease management, rapid, efficient, and safe responses are required to minimize viral threats. The use of virus-resistant crop varieties is a traditional and efficient way to reduce losses caused by viral infections. However, conventional antiviral breeding strategies, even augmented with modern molecular techniques such as quantitative trait locus (QTL) mapping, marker-assisted selection, and whole-genome sequence-based approaches, are slow and laborious and require monitoring of large crop populations over multiple generations [1].

Advances in genetic engineering and plant transformation techniques in the early 1980s enabled the rapid production of virus-resistant transgenic plants [2]. Since then, many research groups have exploited this approach and generated resistance against numerous viruses in many different crops via transgenic expression of various virus-derived or non-viral genes or their fragments [2].

It should be noted that historically, mainstream approaches to engineering plant virus resistance have been driven by a classical view of the central dogma of molecular biology, in which the genetic information contained in DNA is transcribed into mRNA which in turn directs the synthesis of particular proteins, and variations in proteins give rise to different traits. Accordingly, many transgenic plants were generated to express virus-encoded proteins (to potentially interfere with viral functions), with the expectation that any resistance obtained would be protein-mediated. However, in many cases, the resistance had been surprisingly proven to be RNA-mediated. This phenomenon was named RNA silencing or RNA interference (RNAi) [3]. This is a eukaryotic sequence-specific mechanism that controls endogenous gene expression and destroys foreign nucleic acids. RNAi is triggered by the presence of double-stranded RNA (dsRNA) precursors derived from either host plant hairpin RNA structures or viral replicative dsRNA intermediates, which are cleaved into small (18-27 nucleotides, nt) non-coding (nc) interfering RNAs (siRNAs) or micro RNAs (miRNAs), which are associated with some plant proteins to further mediate the amplified sequence-specific inactivation/degradation of foreign (e.g., viral) or endogenous RNAs respectively [4][5][6]. Importantly, siRNAs operate as defenders that directly target their own (invasive) progenitors, whereas miRNAs silence their endogenous gene targets, many of which are involved in developmental processes and stress responses.

While the small siRNAs and miRNAs constitute important classes of regulatory ncRNA, many other types of ncRNAs are known, and all of these form the majority of the transcriptome, given that only about 2% of transcribed RNAs encode proteins [7]. ncRNAs also include well-known classes of RNAs involved in translation such as tRNAs and rRNA, small nuclear RNAs (snRNAs) involved in RNA splicing, and small nucleolar RNAs (snoRNAs) mostly involved in the regulation of posttranscriptional modification of rRNAs [8]. In addition, the plant genome encodes tens of thousands of long ncRNAs (lncRNAs; >200 nt in length) which include linear and circular lncRNAs with no or little coding capacity [9]. Over the past decade, tens of thousands of novel lncRNAs have been annotated in animal and plant genomes. LncRNAs have emerged as highly important regulatory molecules with a role in plant growth, development, and responses to abiotic and biotic (including virus attack) stresses [9]. However, their precise molecular functions in diverse biological processes are only just starting to be elucidated.

Thus, while the central dogma of DNA>RNA>protein still holds true, ncRNAs can greatly influence this information flow and are, therefore, now an emerging major new source of next-generation targets which may be exploited in diverse applications, including conferring plant resistance against pathogens and viruses in particular [8].

RNA technologies provide us not only with new sources of resistance but also with powerful tools to manage plant defense responses. With regards to antiviral resistance, RNAi-based approaches have long been used in the form of transgene-induced RNA silencing to target the degradation of diverse viral RNAs or to inactivate virus susceptibility genes in various crops [2][10][11][12]. However, more recently, exogenous applications of dsRNAs, siRNAs, and hairpin RNAs have been developed and exploited to trigger viral RNA degradation, which could be considered more sustainable, safe, and publicly acceptable than transgenic technology [13]. Another breakthrough has come with the development of RNA-guided CRISPR-Cas genome editing technologies which have been applied in plant virology [14][15][16]. CRISPR-Cas or clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated genes (Cas) is a prokaryotic defense system that evolved to eliminate foreign plasmids or viral DNA and has been reprogrammed into genome editing technology in eukaryotic organisms (reviewed in [17]). The CRISPR-Cas genome editing system includes a Cas endonuclease and a single-guide RNA (sgRNA). The sgRNA contains a targeting sequence of approximately 20 nt which defines its specificity and a scaffold for Cas protein binding. The Cas protein guided by sgRNA targeting creates a double-strand break at the target DNA site which is then repaired by cellular machinery. This event is often accompanied by deletions or insertions in the cleaved region of the open reading frame (ORF), which leads to knockout of the target gene. RNA-targeting Cas endonucleases have also been described [17][18][19] and successfully used for engineering plant virus resistance in a way similar to RNAi: either by direct targeting of viral DNA or RNA or by inactivation of plant host genes required for the development of virus infections (reviewed in [14][15][16]. The use of various sequences and the mechanisms of RNA-based engineering plant virus resistance have been considered in detail in a number of reviews [14][15][16][20][21][22][23][24][25][26][27][28][29]. However, there has not been a review that has covered all of the approaches used to date.

2. Non-Coding RNAs and Plant Antiviral Defense

2.1. siRNAs

Of the different types of ncRNAs, siRNAs have attracted considerable attention due to their direct targeting of viral RNAs for degradation as a part of the natural RNAi-based antiviral immune response [4][30]. For RNA-containing plant viruses, the formation of siRNA is initiated by the Dicer-like enzyme (DCL), which cuts virus-specific dsRNAs (which often arise as intermediates in virus replication) into short siRNA fragments of 21–25 nt. One of the two chains (guide strand) of each duplex RNA fragment is then incorporated into a multi-protein RNA-induced silencing complex (RISC) which includes Argonaute (AGO), an endonuclease. This complex facilitates the pairing of the guide strand with a complementary sequence in viral RNAs which is then cleaved by the AGO component [4][5][31][32][33][34]. The resulting “aberrant” viral RNA cleavage products are amplified by endogenous plant RNA-dependent RNA polymerases (*RdRp*), and serve as secondary siRNAs that systemically spread throughout the plant; which is crucial for efficient RNAi-based antiviral defense [35]. RNA silencing may further be enhanced via methylation of the siRNAs by the small RNA methyltransferase HUA ENHANCER1 (HEN1) [34], which serves to improve the silencing signal by promoting siRNA stability and longevity.

For DNA-containing plant viruses (e.g., geminiviruses) the precursors of siRNAs are presumably produced by DNA-dependent RNA polymerase II (Pol II) which mediates bidirectional transcription in both sense and antisense orientations on the viral DNA. Such transcripts can pair virus-specific mRNAs and hence form perfect dsRNAs to be processed by some DCLs into siRNAs [36].

To successfully overcome siRNA defenses and infect plants, viruses have evolved suppressors of RNA silencing (VSRs) which may target key steps in the siRNA pathways by inhibiting siRNA production, sequestering siRNAs, or preventing the spread of RNA silencing signals [37]. Some other viruses such as red clover necrotic mosaic virus may evade silencing by adopting viral RNA structures that are incompatible with the host plant siRNA potentiation machinery [38].

Thus, siRNA-triggered RNAi represents a robust host defense mechanism against plant viruses which has been proven for numerous viruses in many plant species and crops [13][20]. With increasing knowledge of this mechanism, siRNA-based technologies have been emerging as an innovative approach with which to control plant viruses in practical agriculture.

2.2. miRNAs

Fundamentally, miRNAs and siRNAs share some similarities in size, structure, and functions. Both are short duplex RNA molecules that may exert gene silencing effects at the RNA level and are derived from double-stranded regions of RNA precursors. They both require DCL and AGO proteins for processing, maturation, and action, although the species of DCL

and AGO may be different. However, there are many important differences between miRNAs and siRNAs. A major distinction between them is that siRNAs are processed from foreign dsRNAs (such as those derived from transposons, transgenes, or viruses) whereas miRNAs are derived from endogenous precursor transcripts containing double-stranded (typically hairpin) regions encoded by miRNA genes (MIR genes). miRNAs are derived from longer, primary transcripts termed pri-miRNAs. The pri-miRNAs contain an RNA hairpin in which one of the two strands includes the mature miRNA fragment. This hairpin then is cut out by a microprocessor enzyme complex which includes DCL1 nuclease (a member of an RNase III endonuclease family) and HYL1, a dsRNA binding protein [21][39], which act to generate precursor miRNA (pre-miRNA). The pre-miRNA is further cleaved to generate a short RNA duplex in which one strand is the mature miRNA. The mature miRNA is methylated by HEN1 and taken up by the RISC complex to further potentiate RNAi. Most plant miRNAs typically have perfect or near-perfect complementarity with their targets. This is consistent with their primary mode of action being cleavage of target mRNAs. However, some plant miRNAs and their natural targets may have several mismatches, which while they do not initiate cleavage, may promote repression of mRNA translation [39].

Many miRNAs are evolutionarily conserved across major taxa of the Kingdom *Plantae* from mosses to monocots and eudicots and can be grouped in two classes: one which is conserved and ancient, and one which consists of miRNAs that are less conserved and younger [22][40]. *In toto*, miRNAs constitute a highly diverse set of molecules which may match nearly all RNAs encoded in a plant genome, thereby controlling almost any aspect of the plant life cycle; playing key roles in growth and development, signal transduction, innate immunity, and responses to various ecological biotic and abiotic stresses [22].

With regards to virus infections, information on miRNAs has mainly focused on their role in the induction of viral disease symptoms and antiviral defense mechanisms. It is well documented that some viruses may control accumulation rates of miRNAs involved in plant symptom development [21]. For example, the severe strain of cucumber mosaic virus (CMV)—Fny suppressed the accumulation of miR159 in *Arabidopsis* plants, thus elevating the accumulation of its gene target transcripts MYB33 and MYB65 (which encode transcription factors that are involved in gibberellin signal transduction). This consequently led to the production of severe viral symptoms [41]. Similar findings were also observed with CMV-LS infection, which resulted in reduced accumulations of miR159, miR165, and miR166 which operate as negative regulators of symptom development [41]. It has also been reported that miR171 may be downregulated by rice streak virus (RSV) infection, which was implicated in reduced chlorophyll content and leaf yellowing [42]. Interestingly, while miRNAs would be expected to contain similarities with host genes and alter their expression via the RNA silencing machinery, it is also possible that siRNAs derived from viral genomes may contain occasional similarities to host genes and potentiate silencing of those genes. For example, siRNA derived from the CMV Y-satellite RNA was found to target the chlorophyll biosynthetic gene (Chll), causing Chll mRNA silencing, which induced yellowing symptoms in tobacco plants [43]. Some of the siRNAs derived from potato virus Y (PVY) are completely complementary to the gene encoding translationally controlled tumor protein (TCTP). This protein is required for successful infection of tobacco by PVY and hence, its targeting by PVY-specific siRNA effectively knocks the protein down and blocks invasion [44]. Why the virus forms siRNA that has an unfavorable impact on itself remains unclear.

Many components of the antiviral silencing machinery such as *AGO or RdRp* genes, which are involved in siRNA biogenesis and activity, are themselves natural targets of various plant miRNAs. For example, the antiviral *AGO1* is the target gene for miR168. To prevent degradation of *AGO1* transcripts and thereby alleviate *AGO1*-mediated antiviral defense, plants have evolved a decoy protein gene *AGO18* [45]. *AGO18* transcripts compete with *AGO1* transcripts for miR168 to attenuate the cleavage of *AGO1* mRNA. Expression of *AGO18* in rice was shown to be activated upon RSV infection, conferring resistance against this virus through partial miR168 sequestration [45]. miR444 has been found to promote antiviral resistance against RSV by enhancing the *RdRp1*-mediated RNA silencing pathway in rice by targeting *RdRp1*-inhibiting proteins [46].

It has also been shown that some miRNAs can target genes that trigger antiviral resistance [47]. For example, miR6019 and miR6020 target mRNA of tobacco *N* gene encoding the resistance TIR-NB-LRR protein gene that mediates hypersensitive response against tobacco mosaic virus (TMV). Overexpression of these miRNAs decreases levels of TIR-NB-LRR transcripts and attenuates *N*-mediated resistance to TMV, suggesting that miR6019 and miR6020 play an important role in controlling resistance to TMV [48][49].

General plant responses contributing to antiviral resistance are often orchestrated by the rapid production of reactive oxygen species (ROS) and several phytohormones such as salicylic acid (SA), jasmonic acid (JA), and auxin [50][51]. It has been demonstrated that ROS- and phytohormone-triggered signaling pathways can be regulated by miRNAs, and these can in turn be influenced by virus infection. For example, miR528 can negatively regulate ROS levels by cleaving L-ascorbate oxidase (AO) messenger RNA [52], thereby reducing AO-dependent accumulation of ROS. Upon RSV infection, miR528 is sequestered by *AGO18*, resulting in increased AO activity and elevated ROS accumulation; rendering plants

more resistant to the virus [52][53]. miR319 which reduces expression of target gene *TCP21* and JA concentrations in rice had elevated abundance after infection by rice ragged stunt virus, leading to a more virus-susceptible phenotype [54]. Recently, transgenic overexpression of mutant miR393 in rice was shown to confer higher susceptibility to rice black-streaked dwarf virus infection (RBSDV), by suppressing the auxin receptor TIR1; suggesting that auxin signaling plays an important role against RBSDV infection in rice [55].

As exemplified by these case studies, miRNAs are currently regarded among the most important gene regulators. As discussed, these small RNAs are involved in many pivotal aspects of plant-virus interactions and are emerging as the next generation targets for engineering plant virus resistance.

2.3. LncRNAs

Another group of non-coding RNAs playing important roles in many biological processes is lncRNAs. LncRNAs are defined as transcripts longer than 200 nt with little or no coding potential. Similar to coding mRNAs, most lncRNAs are transcribed by Pol II. They may also have typical mRNA-like structures such as 5′m^7 G cap, 3′ poly (A) tail, and exon-exon junctions [7][9][56]. In addition, the two plant-specific RNA polymerases—Pol IV and Pol V—can produce non-polyadenylated lncRNAs and hundreds of such transcripts were induced in *Arabidopsis* under stress conditions [57][58]. In general, lncRNAs are typically less sequence conserved and abundant than mRNAs but have greater tissue specificity.

Based on genomic origin, lncRNAs can be classified as intergenic, intronic, or exonic regions in sense or antisense orientation [59]. Besides these widely discussed lncRNAs types, short-lived medium-length lncRNAs (from 200 to 2000 nt), such as those derived from promoter upstream transcripts (PROMPTs) and enhancer RNAs (eRNAs) were also identified in plant and animal cells [60][61]. Although most functional plant lncRNAs have not yet been well characterized, they have been implicated in multiple mechanisms to control a diverse range of gene expression pathways [7][9][56]. LncRNAs may regulate the expression of the genes present on the same locus in *cis* and that of distant genes in *trans. Cis*–acting regulatory lncRNAs modulate the expression of the neighboring target genes either by recruitment of Pol II or through the event of getting transcribed, which results in local chromatin conformational changes that facilitate downstream mRNA transcription [7].

Trans-acting lncRNAs may act as scaffolds to facilitate the recruitment of protein(s) and the formation of RNA protein complexes. Such interactions are usually mediated by specific lncRNA domains and may regulate gene expression either through sequestering proteins and preventing them from reaching their target (as a decoy) or by delivering them to specific target sites (as a guide) [7][9]. LncRNAs may also bind miRNAs (as target mimics) to sequester miRNAs, which disrupts their activity to silence specific mRNAs. Another emerging function of lncRNAs is to control the epigenetic state of particular genes by regulating DNA and histone methylation [62]. For example, certain lncRNA can guide DNA methyltransferase-containing complexes to target genomic loci for methylation and transcriptional repression. In contrast, some other lncRNAs recruit histone methyltransferases to activate gene expression by promoting histone methylation. Depending on the mode of action in the transcriptional or posttranscriptional control of gene expression, lncRNAs can reside either in the nucleus or cytosol [63]. The subcellular localization of lncRNAs is controlled via intrinsic RNA sequence localization elements and/or through their interaction with different binding proteins.

The repertoire of biological processes and mechanisms of action of the lncRNAs are rapidly growing. Plant lncRNAs are known to play pivotal roles in the regulation of flowering time, modulation of reproductive organ development, leaf development, auxin signaling, photomorphogenesis, and responses to biotic and abiotic stresses [63].

It is conceivable that lncRNAs may also play important roles in plant-virus interactions. Indeed, it has been shown that both RNA-containing tobacco rattle virus [64] and DNA-containing tomato yellow leaf curl virus (TYLCV) [65][66] significantly change the pattern of lncRNA accumulation upon virus infection. Moreover, some tomato lncRNAs have been shown to act as competing endogenous target mimics for miRNAs in response to TYLCV, suggesting a role in the regulation of virus resistance in the tomato [65].

The ELENA1 lncRNA identified in another recent study was shown to take part in controlling plant defense by modulating the SA pathway [67]. It was found that ELENA1 is able to interact with fibrillarin, the major protein of the nucleolus which also acts as a negative regulator of plant immunity [68][69]. This interaction with ELENA1 evicts fibrillarin from a complex with MED19a, rendering the MED19a active, which subsequently drives the transcription of SA-inducible immune—responsive (pathogenesis-related) genes [67]. Taken into account that fibrillarin has also been implicated in interactions with plant viruses [68][69], we can anticipate that ELENA1 and/or some other lncRNAS, may play a regulatory role in controlling plant antiviral defense responses.

Interestingly, the citrus Tristeza virus (CTV) encodes its own subgenomic lncRNA, LMT1 [70]. Infection of *Nicotiana benthamiana* with a CTV isolate deficient in LMT1 production (CTV-LMT1d) led to an elevated SA accumulation (which typically enhances virus resistance [71] and a consistent reduction in susceptibility to the virus in comparison to the wild type virus [70]. This study also reported that ectopic expression of LMT1 RNA could suppress SA accumulation and subvert the low-infectivity phenotype of CTV-LMT1d; suggesting that LMT1 promotes plant immune evasion by suppressing SA accumulation [70]. Further studies are warranted to elucidate mechanisms underlying the role of lncRNAs in plant virus infections.

Circular RNAs (circRNAs) are a group of endogenous non-coding ssRNAs that have a closed-loop structure [72]. CircRNAs are generated in the back-splicing process from pre-mRNAs, in which the 5′- and 3′-ends are joined by covalent bonds. Typically, circRNAs exhibit a much higher degree of conservation than linear lncRNAs, but their abundance is low. Due to the existence of similar miRNA binding sites in both circRNAs and mRNAs, many circRNAs may interfere with miRNA-mRNA interactions; indicative of them playing a broad regulatory role in various processes in plants such as growth, development, reproductive processes, biotic and abiotic stress responses [72]. Expression of circRNAs in plants is often promoted by different ecological stresses such as heat, drought, chilling, or pathogen attack. With regards to virus infections, circRNAs have been predicted to function as negative regulators of defense responses to TYLCV infection in tomato [65] and maize Iranian mosaic virus infection in maize. Interestingly, viroids and some viral satellite RNAs are also single-stranded circular RNAs; but in contrast to host circRNAs, they are replicative and infectious or associated with virus infections (able to spread from cell-to-cell as well as from plant to plant [73].

2.4. Small Peptides in lncRNAs

Despite their definition as non-coding RNAs, a growing number of reports have suggested the existence of stably expressed and functional peptides (or microproteins) translated from lncRNAs [74]. These peptides encoded by small open reading frames (smORFs-encoded peptides [SEPs]) are shown to regulate a diverse range of cellular processes in plants and animals [75][76]. Various computational tools and experimental approaches such as ribosomal profiling and mass-spectrometry analysis have recently been explored and exploited to differentiate between coding and non-coding RNAs and to identify ORFs that encode SEPs [74][77]. Besides lncRNAs, pri-miRNAs may also encode SEPs (miPEPs), including miPEP165a from *Arabidopsis,* miPEP171b from *Medicago* [78], and miPEP156a which is evolutionarily conserved in *Brassicaceae* [79]. These peptides presumably modulate the expression of their corresponding miRNAs and activate target genes responsible for tissue and organ development.

There is a growing body of evidence that SEPs play an important role in animal cell immunity [80], suggesting the existence of SEPs with similar functionality in plants. Although the role of SEPs produced by various lncRNAs in plant-virus interactions has not been investigated so far, this function remains a distinct possibility given that novel peptide-based regulators were discovered in the cluster of lncRNAs/circRNAs, which control the animal antiviral response [81]. Future studies on the effect of such small peptides on plant-virus interactions would present a challenging task.

2.5. Other Non-Coding RNAs

As mentioned above, other classes of non-coding RNAs include rRNAs, tRNAs, snRNAs, and snoRNAs. There are no doubts that some functional crosstalk may exist between various activities of these RNAs and virus infections. A striking example of such interplay is that sophisticated functional mimics of tRNAs (transfer RNA-like structures) are found at the 3′-ends of the genomes of some plant positive-strand RNA viruses [82], which may compete for host tRNA binding factors. Viral RNAs may also compete with rRNAs for some ribosomal proteins [83]. These observations may contribute to a better understanding of mechanisms underlying plant-virus interactions and provide new platforms for virus control. However, at present, the involvement of tRNAs, rRNAs, snRNAs, and snoRNAs as targets in plant antiviral responses with RPs is largely unknown. Therefore, exploring antiviral strategies based on the use of these RNA classes is outside the scope of this review article and will likely be the focus of future research.

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