The role of plant small RNAs in NB-LRR regulation

June Hyun Park and Chanseok Shin

Abstract

Small RNAs constitute a fundamental layer of gene regulation for diverse biological processes in plants, including development, metabolism and stress responses. With the advance of high-throughput sequencing technologies and the rapid accumulation of transcriptomic data, the scope of regulation afforded by small RNAs has expanded to encompass plant innate immune responses. Plants have evolved the capacity to control the infection through intracellular surveillance proteins of the nucleotide binding site–leucine-rich repeat (NB-LRR) family that recognize pathogen-encoded effectors and initiate effector-triggered immunity. Emerging evidence indicates that plants have evolved to use specific microRNAs that target conserved domains of NB-LRR-encoding genes and trigger the production of a phased array of 21-nucleotide secondary small interfering RNAs to amplify the silencing effect. Herein, this review describes recent advances in understanding the roles of small RNAs in NB-LRR regulation that provide new insights into small RNA-mediated arms race between plants and their pathogens and discuss the unresolved questions and the future prospects for research on this topic.

Key words: plant innate immunity; NB-LRR protein; microRNA; small RNA

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs that play regulatory roles by negatively affecting gene expression at the posttranscriptional level through mRNA degradation [1] or translation repression [2–4]. Extensive studies have shown that miRNAs regulate numerous biological processes in plants, including development, metabolism, hormone signaling and stress responses [5]. Plant miRNAs are derived from the distinct noncoding transcripts of miRNA genes transcribed by RNA polymerase II. The primary miRNAs (pri-miRNAs), which form a secondary fold-back structure, are consequently processed by the RNase III-type enzyme Dicer-like 1 (DCL1) to create the precursor miRNAs (pre-miRNAs) [6]. These pre-miRNAs are further cleaved by DCL1 in the nucleus into miRNA duplexes, which are stabilized by 2’-O-methylation catalyzed by Hua Enhancer 1 [7] and transported to the cytoplasm by HASTY [8]. One strand (the passenger strand) of the miRNA duplexes is often discarded by unwinding or cleavage [9], and the other strand (the guide strand) is retained in the RNA-induced silencing complex (RISC). The guide strand specifies target recognition by RISC, which negatively regulates target gene expression [6]. Plant miRNAs mostly mediate the cleavage of target miRNAs with near-perfect complementarity, exerting a substantial effect on gene expression [5].

The recent past few years have seen rapid progress in our understanding of small RNAs in plants. The existence of gene families encoding the RNA silencing machinery, including RNA-dependent RNA polymerase (RDR), Dicer-like (DCL) and Argonaute (AGO) proteins in plants, indicates the operation of multiple small RNA-directed silencing pathways. Small interfering RNAs (siRNAs) are a complex pool of small RNAs in plants and derived from near-perfect double-stranded RNA (dsRNA) precursor, which are generated either from antisense transcription or by the action of RDRs. Small RNAs have been classified based on their origin and biogenesis; trans-acting small interfering RNAs (ta-siRNAs), natural antisense transcript-derived small interfering RNAs and heterochromatic small interfering RNAs...
Plant immune response

Unlike animals, plants do not have mobile defender cells and a somatic adaptive immune system. The plant innate immune system is an ancient and evolutionarily conserved defense strategy [21]. This can be considered as two interconnected tiers of receptors, one outside and the other inside the cell [21]. The first tier comprises the transmembrane pattern recognition receptors that recognize slowly evolving pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns, such as flagellin and lipopolysaccharides [22]. Recognition triggers a general defense response referred to as PAMP-triggered immunity (PTI), which requires signaling through MAP kinases cascades and WRKY transcription factors [23].

In a co-evolutionary arms race between pathogens and their host plants, pathogens have counteracted PTI by introducing effector molecules into host cells, which promotes virulence and results in effector-triggered susceptibility (ETS) [24]. Host plants, in turn, have acquired resistance (R) proteins (i.e. NB-LRR proteins) that recognize specific effectors and elicit a secondary immune response called effector-triggered immunity (ETI) [21]. This is an amplified PTI response that typically results in hypersensitive cell death at the infection site [21]. These intracellular disease R proteins play a vital role in detecting pathogen effectors (avirulence proteins) by recognizing effector-induced modifications to other host proteins [25]. R proteins are generally characterized as having a nucleotide-binding site (NB) domain and a C-terminal leucine-rich repeat (LRR) domain [26].Plant genomes often contain up to several hundred NB-LRR effector-like proteins [26, 27] that can be further classified into two major subfamilies defined by the presence of Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) motifs in the N-terminal domain [28–30]. For detailed reviews of NB-LRR proteins, please see [28–30].

The preliminary observations of small RNA function in NB-LRR regulation

In 2007, Yi et al. reported the first investigation of the potential effects of posttranscriptional silencing in dampening excessive R gene expression at complex loci in Arabidopsis [31]. The Arabidopsis RPP4 locus consists of seven TIR-NB-LRR class-R genes. Two genes at this locus named RPP4 and SNC1 confer resistance to the bacterium Pseudomonas syringae [32, 33] and the oomycete Hyaloperonospora parasitica [34], respectively, and they are coordinately regulated by small RNA-directed silencing [31]. The endogenous small RNAs generated by antisense transcription from SNC1 transcripts seem to repress the members of this cluster. Mutants defective in small RNA biogenesis, such as dcl4 and ago1, showed an increased accumulation of SNC1, a well-characterized TIR-NB-LRR gene [31].

Miniature inverted repeat repeatable elements (MITEs) are considered to serve as major evolutionary elements in transposon-mediated gene regulation in plants by producing small RNAs [35, 36]. Genome-wide analysis of MITEs in Solanaceae suggested that Mis (MITEs in Solanaceae) small RNA contributes to regulation of tobacco mosaic virus (TMV) R gene expression posttranscriptionally [37]. In addition, several studies, mainly focusing on the genome-wide identification of miRNAs in plants, have reported the 5’ RACE analysis of miRNA-directed cleavage of NB-LRRs in Brassica rapa [38], Vitis vinifera [39], Pinus taeda [40] and Citrus trifoliata [41], suggesting that small RNAs might play a general role in NB-LRR regulation.

Identification of miRNAs targeting NB-LRRs in Solanaceae

More recent findings have provided strong evidence that NB-LRR transcripts are regulated by miRNAs at several conserved motifs [42–44]. The N gene [45], encoding a TIR-NB-LRR type protein that confers resistance to TMV, was cleaved by nta-miR6019 and nta-miR6020 [43]. Transient overexpression of these miRNAs in Nicotiana benthamiana attenuated N gene-mediated resistance to TMV and resulted in the reduction of the hypersensitive response, indicating that these miRNAs are important for plant immunity [43]. A large-scale investigation of NB-LRR-targeting miRNAs in tomato identified the miRNA superfamily of miR482/2118, suggesting their possible conserved role for defense responses [42]. The members of the miR482 family are unique in that they are 22 nt in length rather than 21 nt, they have unusually variable sequences compared with other miRNA families and they are often highly expressed [42]. Of the 186 NB-LRR proteins annotated in the tomato genome, 58 were predicted to be miR482 targets, a high proportion of which were CC-type NB-LRR proteins [42]. Although some miR482 isoforms were predicted to target a unique NB-LRR, most miR482 members in tomato have multiple NB-LRR targets encoding a variant of the P-loops and Walker A motif, a highly conserved signature structure of R proteins [42]. The various miR482 isoforms are complementary to the wobble nucleotide position of the conserved amino acids in this motif and thereby enable only a few miRNAs to regulate large NB-LRR-coding gene repertoires [42].

Phased secondary siRNAs are derived from NB-LRR transcripts

Previous studies reported that miRNAs of 22 nt in length are the potential triggers of the production of secondary siRNAs, which
are often observed in a 21 nt phased interval [46, 47], analogous to tasiRNA in Arabidopsis (Figure 1). In tomato, there were 15 phased siRNA generating loci, six of which corresponded to NB-LRRs [42]. For example, miR482 directed the cleavage of LRR1 mRNA, a CC-NB-LRR type protein, which defined the first position in the phased registers and initiated production of phased siRNAs [42].

Similarly, Li et al. showed that cleavage by nta-miR6019 contributed to increased accumulation of RDR6 and DCL4-dependent biogenesis of phased siRNAs [43]. In addition, RNA secondary structure analysis showed that these miRNA precursors have an asymmetric bulge owing to a nucleotide mismatch in the hairpin structure that may lead to accumulation of 22-nt miRNAs [43], supporting the model that DCL-1 mediated biogenesis of 22 nt miRNAs is dependent on the asymmetric bulge in the precursor structure [48]. Although the functional relevance and exact contribution of individual phased siRNA in target silencing is not fully understood, these phased siRNAs might act synergistically with miRNAs, leading to enhanced and amplified regulation of NB-LRR transcripts (Figure 1).

**Biological meaning of small RNA-mediated regulation of NB-LRRs in Solanaceae**

What is the biological significance of small RNA-mediated NB-LRR regulation? The constitutively high level of NB-LRR-targeting miRNAs raises the intriguing possibility that NB-LRR transcripts are lowly expressed in the absence of pathogens by posttranscriptional regulation. Shivaprasad et al. found that expression level of miR482 decreased in tomato plants infected with viruses, such as turnip crinkle virus, cucumber mosaic virus and tobacco rattle virus, that resulted in upregulation of NB-LRR transcripts [42]. Similarly, the infection of bacterial pathogen P. syringae DC3000 reduced the level of miR482 [42]. Although the ETI response is generally specific for some but not all strains of pathogens [49], the overexpression of NB-LRR proteins can induce defense responses, independently of protein-based recognition of pathogen effectors. Because the relieved silencing of NB-LRR transcripts resulted in a 2- to 3-fold increase of NB-LRR transcripts, Shivaprasad et al. suggested that miRNA-targeted NB-LRRs might be involved in non-race-specific resistance against viral and bacterial pathogens [42].

Based on these analyses, it is plausible to speculate that small RNA-mediated NB-LRR regulation is critical for plant immune responses (Figure 2A and B). However, there is no clear example of a pathogen interaction that specifically suppresses miRNAs, which causes global and coordinated changes in NB-LRRs and phased siRNAs. Although Li et al. showed that co-expression of N gene with nta-miR6019/6020 attenuated N-mediated resistance to TMV [43], which, however, did not reflect the endogenous regulation of defense responses. In addition, the expression of these miRNAs was barely detected by northern blotting in wild-type plants [43], suggesting that the in vivo contribution of miRNAs participating in defense responses might be less physiologically relevant. Shivaprasad et al. showed that miR482-directed silencing of a few NB-LRRs of unknown function was relieved on the infection of viral and bacterial pathogens [42], but they did not demonstrate the direct consequences of induced NB-LRR transcripts for defense responses (i.e. decrease in NB-LRRs resulting from miR482 overexpression reduces a hypersensitive response in infected plants, whereas induction of NB-LRRs in the absence of miR482 leads to an enhanced hypersensitive response). Therefore, as yet, one cannot definitively conclude that pathogens mediate the suppression of specific miRNAs and thereby increase NB-LRR expression, which in turn improves specific defense responses.

Plant genomes contain up to thousands of genes related to defense responses that offer protection from various pathogens. Small RNA-mediated attenuation of NB-LRR transcripts could have co-evolved with various NB-LRR loci. Depending on the circumstances and pathogenic challenges, the shift in the balance between the costs and benefit would drive the variation in sequence and expression of these small RNAs [42]. Shivaprasad et al. described that these small RNAs could minimize the overall metabolic cost of producing disease resistance proteins because NB-LRR transcripts would be expressed at low levels without a pathogen challenge [42]. This would create a background in which there could be a diversification and amplification within the R gene family without there being an undue burden on plants (Figure 2A and B).
Figure 2. The possible role of small RNAs in NB-LRR regulation. (A) Constitutively high level of expression of miR482 allows the simultaneous silencing and a steady-state regulation of the NB-LRR transcripts, which could be further amplified by phased siRNA-mediated silencing. The evolution of this tight control system might have been favored by reduction of the fitness cost. (B) In the presence of pathogens (e.g. virus, bacteria and oomycete), small RNA-mediated silencing of NB-LRRs is relieved (possibly via RNA silencing suppression by pathogens), leading to the accumulation of NB-LRRs for enhanced defense responses. The classic zigzag model of the plant immune system according to Jones and Dangl [21]. Plants recognize pathogen-associated molecular pattern (PAMP, red circles) to trigger PTI to raise defense above the resistance threshold. As a counter defense, pathogens secrete effectors (blue circles) to defeat PTI, resulting in ETS. The effector (so-called avirulence (Avr) factors) is recognized by a corresponding NB-LRR protein (Avr-R complex), activating ETI, an amplified version of PTI that passes an HR threshold, as a counter-counter defense strategy. (C) In the presence of rhizobia, small RNAs might downregulate the NB-LRR transcripts to limit defense response to encourage beneficial microbial interactions. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)
The possible role of RNA silencing suppression by plant pathogens

A battle between plants and their pathogens during co-evolution may involve pathogen-encoded suppressors of RNA silencing. Emerging evidence has shown that viral, bacterial and oomycete suppressors of RNA silencing can hijack small RNA pathways at multiple steps, which could suppress immunity [19, 20, 50, 51]. The oomycete pathogen, Phytophthora sojae, secretes two effectors that suppress RNA silencing by inhibiting the biogenesis of small RNAs, resulting in reduced miRNA and secondary siRNA levels [50]. From the perspective of small RNAs targeting NB-LRRs, it appears to be counterproductive that pathogens would suppress RNA silencing, thereby reducing the levels of small RNAs and increasing NB-LRRs. Qiao et al. pointed out that because these effectors are only expressed late in infection, the increased NB-LRRs might be involved in facilitating the transition from biotrophic to necrotrophic growth in P. sojae by triggering host cell death [50]. Otherwise, on the host side, pathogen-mediated upregulation of NB-LRRs might be a mechanism of counteracting pathogen offense to enhance defense response, which could be one possible explanation of how NB-LRR-targeting miRNAs are downregulated on pathogen infection (Figure 2B).

Lessons from NB-LRR-targeting small RNAs in legumes

Integrated analysis of small RNAs, target prediction and degradome data in legume Medicago truncatula provided some different insights into a role of miRNAs for NB-LRRs in beneficial microbial interactions [44]. Legumes are unique plants because they are able to establish a symbiotic relationship with nitrogen-fixing bacteria called Rhizobia within nodules of their root system, thereby converting inorganic nitrogen gas into nitrogen compounds usable by living organisms [52]. A previous study noted a possible connection between miRNAs and rhizobial symbiosis; miR842, miR1512 and miR3515 were induced on rhizobia infection in soybean roots, and their transgenic expression increased nodule numbers, suggesting that these miRNAs are important for nodule development in soybean [53]. To facilitate beneficial microbial interactions, the defense responses should be limited in the presence of rhizobia, indicating that there might be some regulatory molecules that could globally suppress NB-LRRs. Zhai et al. found that >60% of NB-LRR transcripts in Medicago were identified as potential targets of a few highly abundant 22-nt miRNAs, including miR1507, miR2109 and miR2118, which triggered the production of phased siRNAs [44]. They also systematically analyzed the target cleavage of phased siRNAs by examining degradome data, which identified ~2000 cleavage sites from 570 phased siRNAs, including numerous cases of both cis- and trans- cleavage [44]. It would be therefore expected that these phased siRNAs might be involved in a widespread and interconnected network for controlling NB-LRR transcripts. Because Medicago has relatively higher abundances of NB-LRR-targeting miRNAs than other plant species, Zhai et al. suggested that these are possible candidates for a global suppressor of NB-LRRs to encourage symbiotic interactions with rhizobia [44] (Figure 2C). Supporting this idea, NB-LRR-targeting miRNAs are entirely missing in Arabidopsis, which does not symbiotically interact with rhizobia. However, some plants, such as rice, appeared to be absent of miRNAs targeting NB-LRRs (>500 NB-LRRs present in rice), although they are known to undergo symbiotic interaction [54–56]. In addition, based on expression profiling of different Medicago tissues, these miRNAs are also highly expressed in aerial tissues far from the site of rhizobial interaction [44]. Future experiments and bioinformatics analysis will be necessary to place these miRNAs in a functional pathway of promoting beneficial microbial interactions in plants.

The role of small RNAs in balancing defense response and fitness costs

One of the most attractive hypotheses is that constitutively highly expressed small RNAs in Solanaceae and legumes could provide a steady-state regulation of the NB-LRR transcripts [57] (Figure 2A and B). Plant immune responses should be minimized in the absence of pathogens to prevent autoimmunity that might confer deleterious effects on plant growth, development and yield. Therefore, these small RNAs might play a role of efficient buffers of NB-LRR transcript to a basal level and contributes to the prevention of the fitness costs of overtactive immune responses [57] (Figure 2A and B). In plant genomes, there are numerous NB-LRR genes, and their constitutively high transcription would inflict a large metabolic cost. One could question why plants regulate NB-LRR transcripts posttranscriptionally as opposed to transcriptional regulation? It might be that the regulation of NB-LRRs should be highly flexible so that the level of induction does not result in delays to ETI responses that could be exploited by pathogens. One can postulate that posttranscriptional regulation of NB-LRRs by small RNAs is a subtle mechanism to stabilize the basal expression levels and to provide a means for highly sub-compartment-specific expression, in contrast with the binary mode (on/off) of transcriptional control.

NB-LRR-targeting small RNAs are unusual in that they target highly conserved motifs, thereby governing and limiting the expression of such an extensive gene family, taking evolutionary advantage of the degeneracy of small RNA–target interactions. The miRNA dependency of NB-LRRs might have not been selectively constrained during evolution as target sites could be gained or lost by a few nucleotide substitutions, presumably attributable to either broad or fine-tuning regulation of NB-LRR expression. The feedback regulation between small RNAs and NB-LRRs is also conceivable; NB-LRR-targeting miRNAs would accelerate the triggering of phased siRNA productions in the presence of excessive NB-LRR accumulation that might pass the threshold of autoimmunity (Figure 1). Likewise, self-regulation is possible; small RNAs derived from NB-LRRs could directly regulate their own expression (Figure 1). These types of regulation would prevent unwanted accumulation of NB-LRRs and might act as a buffer to modulate and fine-tune the sensitivity and dynamics of NB-LRRs.

Conclusion and future perspectives

The functional importance of small RNA-mediated regulation of NB-LRRs is not completely understood, yet the latest data undeniably demonstrate that it is widespread especially in plants such as Solanaceae and legumes. Recently, another interesting observation emerged from an in-depth analysis of small RNAs in an evolutionary older gymnosperm species, Picea abies (spruce). This conifer had a surprisingly high proportion of small RNAs derived from NB-LRR transcripts, constituting 48% of all small RNA reads mapping to transcribed sequences. These data suggest that NB-LRR genes are more subjected to small RNA
degradation in conifers [58]. It is therefore tempting to speculate that small RNA-mediated regulation of NB-LRRs is an evolutionarily ancient mechanism of counter-counter defense against pathogens. The family of NB-LRR genes was expanded dramatically in land plants [59], which is in line with observation that NB-LRR-targeting small RNAs are prevalent in high numbers not only in some dicots, but also in a primitive land plant. An important question remains unanswered; this process seems to be prevalent and redundant in some plants, but it is virtually missing in monocots and Arabidopsis [57], indicating a strong evolutionary force that leads to diversifying selection and high levels of variation in plants. Additional studies providing compelling evidence from evolutionary analysis and transcriptomic data are needed to shed light on how this important regulatory system diverged during the evolution of land plants and to clarify the role of these small RNAs in regulating NB-LRRs.

Key points

• MicroRNAs mediate the cleavage of NB-LRR transcripts and trigger the production of phased secondary siRNAs, thereby preventing detrimental accumulation of NB-LRRs.

• Small RNAs might play a role in minimizing the overall metabolic cost of producing disease resistance proteins and serve as a buffer to modulate and fine-tune the sensitivity and dynamics of NB-LRRs.

• The evolution of this tight control system might have been favored by reduction of the fitness cost.

Acknowledgements

The authors apologize to colleagues that space limitations did not allow us to cite all the relevant literatures.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant, funded by the Korea government (MEST; No. 2012R1A2A2A01045528) and the "Next-Generation BioGreen 21 Program for Agriculture & Technology Development (Project No. PJ01115601)”, Rural Development Administration, Republic of Korea.

References