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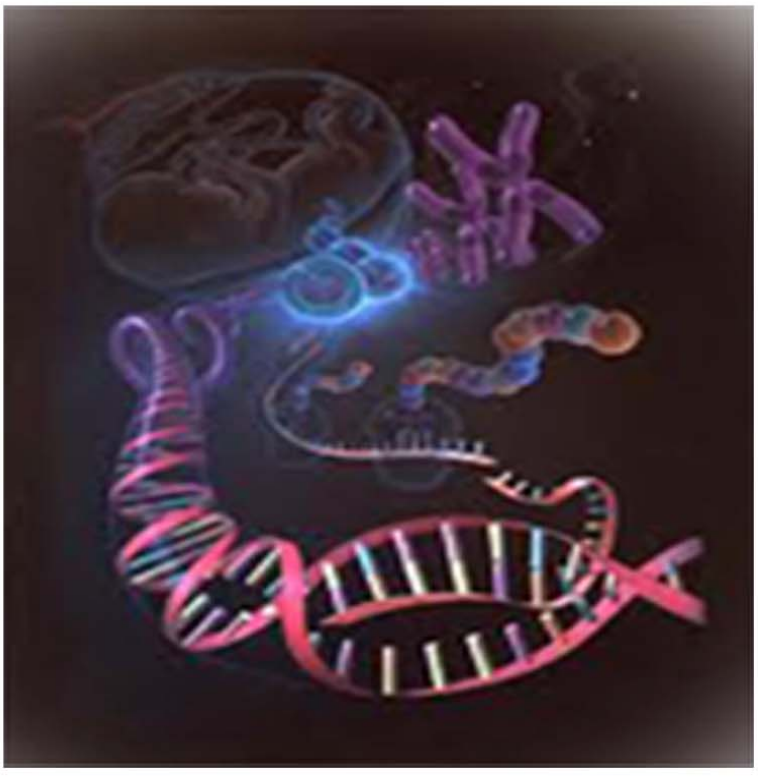

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Review Article

THE REGULATORY ROLE OF SMALL RNAs IN PLANT INNATE IMMUNITY

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Small RNAs are non-coding regulatory RNAs with 20-30 nucleotide (nt) sequences. These plentiful RNAs regulate gene expression at different levels by interfering with mRNA translation. The two different forms of small RNAs are classified into small interfering RNAs (siRNAs) and microRNAs (miRNAs). Small RNAs play a critical role in regulating the interaction of pathogens with plants thereby providing innate immunity to plants. The preliminary interaction between the pathogen and its host is responsible for Pathogen Associated Molecular Pattern (PAMP)-triggered immunity (PTI) in plants. Bacteria counteract PTI by secreting effector proteins into plant cells, which lead to suppression of PTI. Host plants, in turn, have evolved resistance components such as resistance (R) proteins that can recognize effectors and elicit Effector-Triggered Immunity (ETI). Many host miRNAs and siRNAs are induced or suppressed by various pathogen challenges. These pathogen-responsive small RNAs induce posttranscriptional gene silencing by guiding mRNA degradation or translational repression.

Keywords: Small RNAs, siRNAs, miRNAs, PAMP-triggered immunity, Effector-triggered immunity, mRNA degradation

INTRODUCTION

DNA, RNA, and proteins constitute the major building blocks of life. These are interrelated in the central dogma of life with the two main processes, *viz*., transcription and translation. The three main types of 'classic' RNAs mediating protein synthesis are messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). mRNAs are translated into proteins, whereas tRNAs and rRNAs have housekeeping roles during mRNA translation. These three RNAs are coding types of RNAs. There are non-coding RNAs, which are not translated into proteins. They are called small RNAs. Small RNAs are known to regulate various biological processes in plants including development, metabolism, maintenance of genome integrity, immunity against pathogens, and abiotic stress responses. Different studies have been conducted to evaluate the role of small RNAs in plant defense mechanism and they are found to play a critical role in regulating the plant-pathogen interaction.

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WHAT ARE SMALL RNAS?

Small RNAs are 20-40 nucleotide (nt) long noncoding RNA molecules present in most eukaryotic organisms that regulate gene expression in a sequence-specific manner either transcriptionally or posttranscriptionally (Kim, 2005). These small RNAs are derived from double-stranded RNAs (dsRNAs) and they can induce gene silencing through specific base-pairing with the target molecules.

RNA SILENCING MECHANISM

Small RNA-mediated gene silencing has been observed in a number of eukaryotes for almost two decades but the mechanism underlying the silencing mechanism began to be unraveled only recently. Because these phenomena were seemingly unrelated at the time of discovery, they were referred to as several different terms such as RNA interference (RNAi), co-suppression, Post Transcriptional Gene Silencing (PTGS), or quelling. The RNAi pathway was originally recognized in *Caenorhabditis elegans* as a response to dsRNA leading to sequence-specific mRNA cleavage (Fire *et al.*, 1998). It soon turned out that RNAi is not restricted to nematode and can be induced in *Drosophila melanogaster* (Kennerdell and Carthew, 1998) and vertebrates (Elbashir *et al.*, 2002). This discovery had been preceded by the observation of similar phenomena in plants and fungi although the involvement of dsRNA was uncertain at that time. For instance, in petunia, introduction of exogenous transgenes silenced expression of the homologous endogenous loci (Napoli *et al.*, 1990). These phenomena were called RNA interference in animals, PTGS in plants and quelling in fungi. This wide range of silencing pathways is now collectively known as “RNA silencing”.

A Brief Chronology of Landmarks in RNA Silencing

Napoli *et al.* (1990). The first description of PTGS caused by RNA interference was in a plant (Petunia). Introduction of a chimeric chalcone synthase gene into Petunia resulted in reversible co-suppression of homologous genes and created a block in anthocyanin biosynthesis (Figure 1). Romano and Macino (1992) PTGS was subsequently seen in a fungus (*Neurospora crassa*) and was referred to as quelling (Figure 2). Fire *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. The two micro RNAs *viz.*, *lin-4* and *let-7* were found to control the timing of developmental events in the worm inhibiting translation of target mRNAs (Figure 3).

Figure 1: RNA Silencing in Petunia

Wild type RNA silencing

Figure 2: Quelling of *Neurospora crassa*

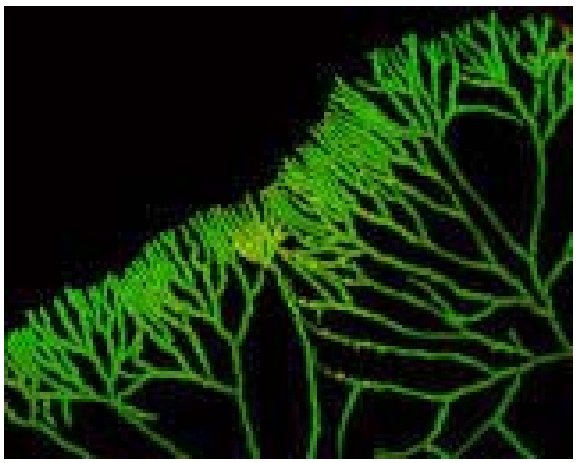
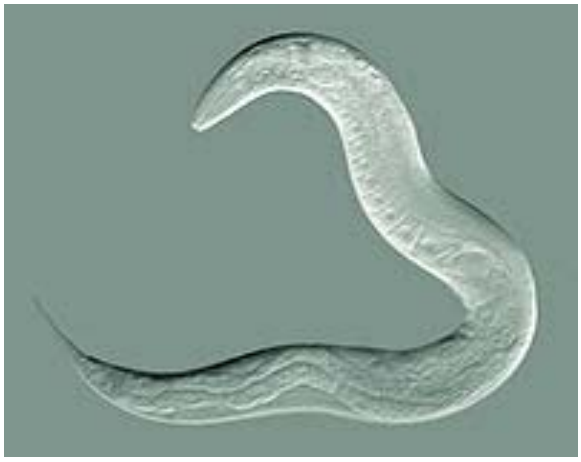


Figure 3: *Caenorhabditis elegans*



Understanding of the cellular processes responsible for PTGS exploded in 1998 with the discovery that it was triggered by double-stranded RNA (dsRNA), a discovery that won Fire and Mello the 2006 Nobel Prize in Physiology or Medicine (Figure 4).

Figure 4: The Nobel Prize In Physiology or Medicine 2006

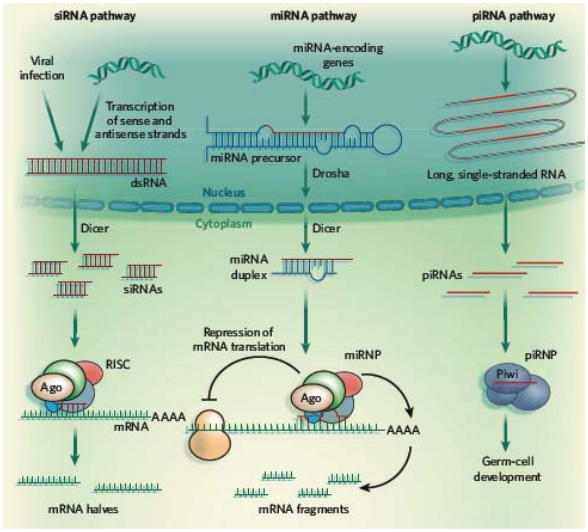
Andrew Z Fire

Craig C Mello

TYPES OF SMALL RNAS

Small RNAs come in mainly three different forms, *viz.*, small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-associated RNAs (piRNAs) (Figure 5).

Figure 5: Formation of Small RNAs



Small Interfering RNAs (siRNAs)

These are small RNAs (20-25 nucleotides in length) formed through cleavage of long double-stranded RNA molecules and by base-pairing of complementary RNAs. An enzyme called Dicer cleaves dsRNA into shorter double-stranded siRNAs that are roughly 20 base pairs long. One siRNA strand then assembles into an effector complex known as RNA Induced Silencing Complex (RISC). This complex uses the siRNA guide to identify mRNAs with a sequence perfectly complementary to the siRNA. RISC then cleaves the mRNA in the middle of the mRNA-siRNA duplex, and the resulting mRNA halves are degraded by other cellular enzymes (Vazquez *et al.*, 2004). Under some circumstances, siRNAs might associate into complexes other than RISC that function in the nucleus and silence gene transcription. siRNAs are particularly important for taming the activity of transposons and combating viral infection, but they can also regulate protein-coding genes. Synthetic siRNAs can also be artificially expressed for experimental purposes.

MicroRNAs (miRNAs)

Those small RNAs (20-25 nucleotides in length) that are encoded by specific genes and function in repressing mRNA translation or in mRNA degradation in plants and animals are referred to as microRNAs. They are processed from long, single-stranded RNA sequences that fold into hairpin structures and contain imperfectly base-paired segments. The processing generally occurs in two steps, and is catalyzed by the enzymes Drosha (in the nucleus) and Dicer (in the cytoplasm). One strand of the resulting miRNA duplex, resembling an siRNA, then incorporates into a RISC-like miRNA-ribonucleoprotein (miRNP) complex. The main components of RISC and miRNPs are proteins of the Argonaute (Ago) family. Depending on the level of complementarity, miRNAs induce mRNA degradation or repress their translation. Unlike the siRNA pathway, miRNA mediated degradation is initiated by enzymatic removal of the mRNA poly (A) tail (Ambros *et al.*, 2003).

Piwi-associated RNAs (piRNAs)

These are small RNAs (25-30 nucleotides in length) that are generated from long single-stranded precursors in a process independent of Drosha and Dicer. They function in association with the Piwi subfamily of Argonaute proteins, and are essential for the development of germ cells.

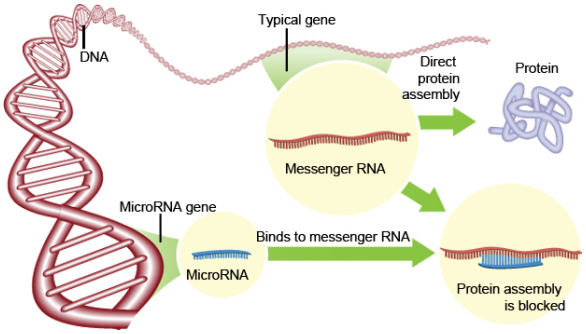
Small RNA Pathways

The pathways of both miRNA and siRNA are described in detail below:

miRNA Pathway

miRNAs are derived from the transcripts of miRNA genes generated by RNA polymerase II. The primary miRNA (pri-miRNA) transcript forms a fold-back structure, which is processed into a stem-loop precursor known as precursor miRNA (pre-miRNA). A protein named DDL has been proposed to play an important role in miRNA biogenesis by recruiting predominantly DICER-like protein 1 (DCL1) to pri-miRNA for downstream processing. The pre-miRNA is acted upon by DCL1 to form the small RNA duplex. The small RNA duplex is then methylated at the 3' ends and is exported to the cytoplasm by an export in homolog. Mature miRNA is preferentially incorporated into AGO1 and guides the complex to the target mRNA for cleavage or translational inhibition on the basis of sequence complementarity (Figure 6).

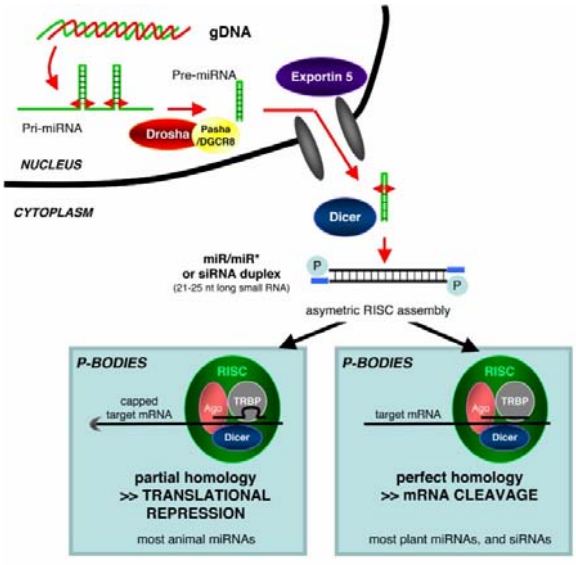
Figure 6: Simplified Representation of Action of miRNA



miRNA genes are transcribed by RNA polymerase II to generate the primary transcripts (pri-miRNAs). The initiation step (cropping) by the Drosha-DGCR8 complex results in pre-miRNAs of ~70-nt, which are exported by the export in protein. Upon export, Dicer participates in the second step of processing (dicing) to produce miRNA duplexes. The duplex is separated and usually one strand is selected as mature miRNAs, whereas the other strand is degraded. The final products act as guide molecules in translational

control or cleavage of certain mRNAs. The figure below shows the detailed representation of miRNA pathway which shows translational repression and mRNA cleavage (Figure 7).

Figure 7: miRNA pathway

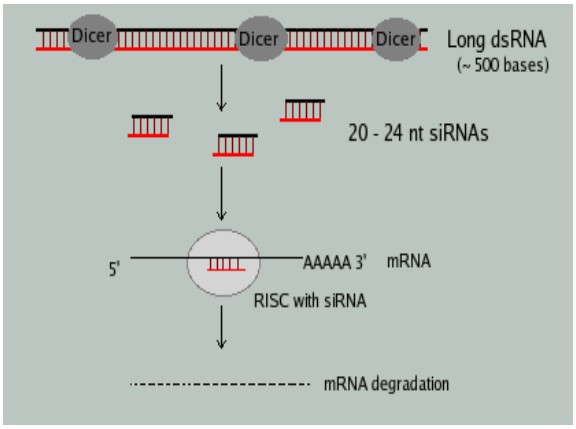


siRNA Pathway

siRNAs have a well-defined structure: a short (usually 21-nt) double-strand RNA (dsRNA) with 2-nt 3' overhangs on either end. Each strand has a 5' phosphate group and a 3' hydroxyl (-OH) group. This structure is the result of processing by dicer, an enzyme that converts either long dsRNAs or small hairpin RNAs into siRNAs. siRNAs can also be exogenously (artificially) introduced into cells by various transfection methods to bring about the specific knockdown of a gene of interest. In essence, any gene whose sequence is known can, thus, be targeted based on sequence complementarity with an appropriately tailored siRNA.

In contrast to miRNAs that are derived from imperfectly base-paired hairpin loop structures, siRNAs are derived from perfectly paired double-stranded RNA (dsRNA) precursors. These dsRNA precursors are derived either from antisense transcription or by the action of a cellular RNA-dependent RNA polymerase (RDR). RNA Pol II transcribes noncoding genes and the long primary transcript products are initially cleaved by miRNAs loaded with RISCs, resulting in a 5' fragment or a 3' fragment. These fragments then act as a template for synthesis of a complementary strand and triggers silencing of the target by destabilizing the target mRNA through decapping and 5'-3' degradation (Figure 8).

Figure 8: Diagrammatic Representation of siRNA Pathway



The Tables given below (Tables 1 and 2) summarize the similarities and differences between siRNA and miRNA.

Table 1: Similarities Among siRNA and miRNA

siRNA	miRNA
Normally processed from long dsRNAs	Processed from 70 nt long stem-loop or hairpin precursors
Dicer is involved in the processing	Dicer is involved in the processing
Usually 22 nt long	Usually 22 nt long

Table 2: Differences Between siRNA and miRNA

siRNA	miRNA
Double stranded with 2nt 3' overhang	Single stranded
High homology with target RNA	Few mismatched nucleotides
Cleavage of target mRNA by RISC	Cause RISC-mediated cleavage or block translation of mRNA
Induced by virus, transposons or transgenes	Endogenously present with different roles in development

PLANT INNATE IMMUNITY

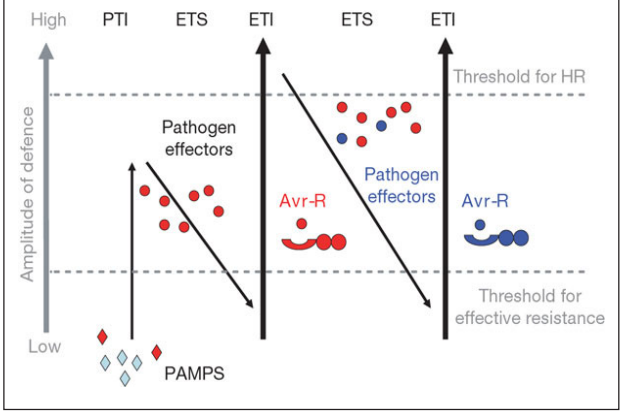
The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. This means that the cells of the innate system recognize and respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host. Innate immune systems provide immediate defense against infection, and are found in all classes of plant and animal life.

As with animals, plants attacked by insects or other pathogens use a set of complex metabolic responses that lead to the formation of defensive chemical compounds that fight infection or make the plant less attractive to insects and other herbivores. Plants, unlike animals, lack mobile defender cells and a somatic adaptive immune system like antibody or T-cells. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites. "Resistance" (R) proteins, encoded by R genes, are widely present in plants and detect pathogens. Many plant R proteins might be activated indirectly by pathogen-encoded effectors, and not by direct recognition. This 'guard hypothesis' implies that R proteins indirectly recognize pathogen effectors by monitoring the integrity of host cellular targets of effector action. The concept that R proteins recognize 'pathogen-induced modified self' is similar to the recognition of 'modified self' in 'danger signal' models of the mammalian immune system.

There are two branches for the plant immune system. One uses trans-membrane Pattern Recognition Receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs) and is referred to as PAMP triggered immunity (PTI). The second acts largely inside the cell, using the polymorphic Nucleotide Binding-Leucine Rich Repeat protein products encoded by most *R* genes. They are named after their characteristic Nucleotide Binding (NB) and Leucine Rich Repeat (LRR) domains. Pathogen effectors from diverse kingdoms are recognized by NB-LRR proteins and activate similar defense responses, this being referred to as Effector Triggered Immunity (ETI). NB-LRR-mediated disease resistance is effective against pathogens that can grow only on living host tissue (obligate biotrophs), or hemi-biotrophic pathogens, but not against pathogens that kill host tissue during colonization (necrotrophs).

The plant immune system can be represented as a four phased 'zigzag' model (Figure 9). In phase 1, PAMPs (or MAMPs) are recognized by PRRs, resulting in PAMP-Triggered Immunity (PTI) that can halt further colonization. In phase 2, successful pathogens deploy effectors that contribute to pathogen virulence. Effectors can interfere with PTI. This results in Effector-Triggered Susceptibility (ETS). In phase 3, a given effector is 'specifically recognized' by one of the NB-LRR proteins, resulting in Effector-Triggered

Figure 9: A Zigzag Model Illustrating Plant Immune System



Immunity (ETI). Recognition is either indirect, or through direct NB-LRR recognition of an effector. ETI is an accelerated and amplified PTI response, resulting in disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site. In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection results in new *R* specificities so that ETI can be triggered again (Chisholm *et al.*, 2006).

Several pathogen-responsive siRNAs play an important role in the ETI pathway. Many recent studies were showing that the plant endogenous small RNAs regulate plant immunity and the pathogens also suppress the small RNA-mediated silencing pathway. Pathogen infection can alter DNA methylation and chromatin modification. DNA methylation and chromatin modification may also be regulated by endogenous small RNAs, which contribute to the evolution of plant immune systems in response to pathogens.

HOST ENDOGENOUS SMALL RNAS IN PLANT-MICROBE INTERACTIONS

Plants have evolved multiple layers of defense in response to pathogen attacks. There is increasing evidence that small RNAs are involved in regulating plant responses to adverse conditions, including biotic stresses (Chellappan *et al.*, 2009). Antiviral defense involving virus-derived small RNAs is an important example of an interaction between plant and pathogen that is mediated by small RNAs. However, these small RNAs are derived from viruses and are therefore exogenous in origin. Unlike viruses that replicate inside the host cell, bacteria, fungi, and other microbes interact with plants without undergoing DNA or RNA replication and transcription inside the plant cell. In these interactions, host endogenous small RNAs play an important role in counteracting these pathogens. Recent reports have shown that plant endogenous small RNAs, including miRNAs and siRNAs, are integral regulatory components of plant defense machinery against bacteria and fungi.

Bacterial Infection

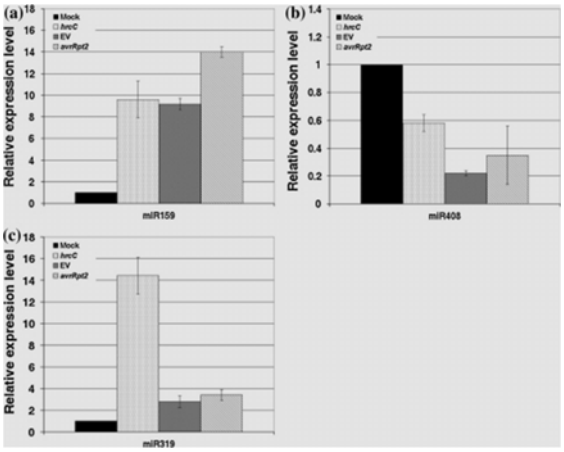
In *Arabidopsis*, the first miRNA discovered to play a role in defense against pathogens was miR393 (Navarro *et al*., 2006). miR393 is induced by a bacterial flagellin-derived PAMP, Flg22. miR393 negatively regulates auxin signaling by targeting auxin receptors TIR1 (transport inhibitor response 1), AFB2 (auxin signaling F-box protein 2), and AFB3. However, a TIR1 paralog, AFB1, was found to be partially resistant to miR393-mediated cleavage because of extra mismatches in the miRNA target site (Katiyar-Agarwal and Jin, 2010).

Transgenic lines expressing AFB1 were more

susceptible to virulent *Pseudomonas syringae* pv. tomato strain DC3000 (*Pst* DC3000) and displayed enhanced disease symptoms (Figure 10). To determine whether miR393 is involved in race-specific resistance, these transgenic plants were inoculated with avirulent *Pst* DC3000 carrying an effector gene, *avrRpt2*. Bacterial growth in transgenic lines expressing AFB1 did not differ from nontransformed plants even at 4 days postinoculation (dpi). These data suggest that miR393 has a role in imparting basal resistance but not race-specific resistance. Induction of miR393 was further confirmed by Fahlgren *et al*. (2006) when they carried out deep sequencing of *Arabidopsis* leaves at 1 h and 3 h postinoculation (hpi) with a nonpathogenic strain, *Pst* DC3000 *hrcC*", which has a mutation in the type III secretion system (TTSS). Relative to uninfected leaves, miR393 was induced ten fold in the infected leaves at 3 hpi. Over-expression of miR393a from a strong constitutive promoter resulted in lower levels of TIR1 mRNA in transgenic lines and these lines exhibited restricted bacterial growth. Besides miR393, two other miRNA families, miR160 and miR167, were also upregulated at 3 hpi. These miRNAs target members of the Auxin-Response Factor (ARF) family of transcription factors that are also involved in auxin signaling. Thus, in response to bacterial infection, plants suppress multiple components of the auxin signaling pathways. The relative expression levels of miRNAs were compared to the mock by quantitative RT-PCR (Figure 11).

Figure 10: Bacterial Speck Disease of *Arabidopsis*

Figure 11: Relative Expression Levels of miRNAs



Note: Mock – control hrcC – Avirulent *Pst* DC3000 *hrcC; EV* - Virulent *Pst* DC3000 with empty vector *avrRpt2 - Pst* DC3000 with an effector.

Fungal Infection

RNA silencing is a robust strategy developed by plants to defend against pathogens, including fungi. Lu *et al.* (2007) tested whether small RNAs are involved in the infection of loblolly pine by the endemic rust fungus, *Cronartium quercuum* f. sp. *fusiforme*. Infection with this fungus causes fusiform rust disease, which is characterized by stem and/or branch galls (Figure 12).

Small RNAs were cloned from the developing xylem of pine, and 26 miRNAs belonging to four conserved and seven loblolly pine-specific miRNA families were identified. Using small RNA

Figure 12: Fusiform Rust Disease on Loblolly Pine

expression profiling, miRNAs involved in disease development were delineated and compared in uninfected pine trees and trees infected with the fusiform rust fungus. The transcript levels for these 11 families of miRNAs were unchanged in roots and in stems above the galls, but transcript levels for 10 of these miRNA families were significantly suppressed in galled stem. These reduced levels of miRNAs in galled stems relative to healthy stems were correlated with increased levels of their target transcripts relative to healthy stems. Interestingly, although the expression of these miRNAs was unchanged in the stem above the gall, their target transcripts were significantly upregulated in stem above the gall as compared with healthy stems. This result suggests that fungal infection at the gall probably immunizes the uninfected stem and may provide protection ahead of the spreading infection. Taken together, these data highlight the complexity of plant-microbe interactions mediated by small RNAs in the galled tissue and the tissue surrounding the gall. The signals responsible for the upregulation of defense responsive genes in the uninfected tissue around the gall remain to be identified.

Viral Infection

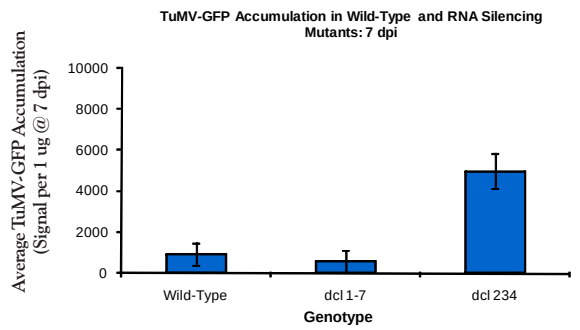
Two miRNAs, miR158 and miR1885, were greatly upregulated when *Brassica rapa* was infected by Turnip mosaic virus (TuMV) (He *et al.*, 2008). The induction of miR158 and miR1885 is highly specific to TuMV infection because infection of *B. rapa* and *B. napus* with Cucumber mosaic virus, Tobacco mosaic virus (TMV), or the fungal pathogen *Sclerotinia sclerotiorum* had no such change (Figure 13). The putative target for miR1885 is predicted to be a member of the TIR-NBS-LRR class of disease-resistant proteins. It is suggested that miR1885 is a novel miRNA generated from gene-duplication events from the TIR-NBS-LRR class of proteins. Understanding the mechanism of plant defense responses against viruses will require the identification of additional miRNAs that are regulated by viral infection.

Figure 13: Turnip Mosaic Virus

Infected Uninfected

The DCL4-dependent virus-derived siRNAs were necessary and sufficient (among the DCL family) to prevent initial infection foci in TuMV-AS9-GFP-inoculated leaves (Figure 14). By contrast, DCL2-dependent siRNAs were neither necessary nor sufficient to limit infections of TuMV-AS9-GFP

Figure 14: TuMV Green Fluorescent Protein Accumulation in wild-type and RNA Silencing Mutants



in either inoculated leaves or cauline leaves. Interestingly, DCL2 limited systemic infection of inflorescence tissues in the absence of DCL4 Why, in the absence of DCL4, is DCL2 sufficient to prevent viral infection in inflorescences but not in inoculated leaves? It is likely not due to differential expression, as both of DCL4 and DCL2 are expressed at similar levels in both leaf and inflorescence tissues. These results also cannot be explained by differential access by DCL2 to dsRNA substrates because 22-nucleotide-long TuMV-GFP- derived siRNAs were detected both in leaves and in inflorescence of dcl4-2 single mutant plants. A quantitative DCL2-mediated reduction of local viral accumulation resulting in limited systemic spread is also unlikely, as DCL2 alone had no effect on suppressor-deficient virus accumulation in inoculated leaves (He *et al.*, 2008).

CONCLUSION

More and more studies have shown that many host miRNAs and siRNAs are induced or suppressed by various pathogen challenges and that modulation of miRNA and siRNA levels plays an important role in gene expression reprogramming and fine-tuning plant responses against a wide range of pathogens. These pathogen-responsive small RNAs induce posttranscriptional gene silencing by guiding mRNA cleavage/degradation or translational repression, or may guide transcriptional gene silencing by direct DNA methylation or chromatin modification. This idea is supported by observations that many components in the small RNA pathways are required for plant defense responses and immunity. As a countermeasure, viruses and bacteria have developed VSRs and BSRs to suppress host RNAi machinery and compromise disease resistance in plants. To combat continuously evolving pathogens, plants have also evolved components, such as R proteins, that can recognize VSRs and BSRs and trigger robust and rapid resistant responses, which are referred to as ETI. The study of small RNA-mediated regulatory mechanisms in plant immunity is an emerging field, and we expect that many more pathogen-responsive small RNAs will be identified using new technologies, such as high throughput deep sequencing. Characterization of these small RNAs and their target genes will reveal new components in plant resistance signaling pathways and help us understand the molecular mechanisms of plant immunity. We also expect that more silencing suppressors will be identified from viruses, bacteria, fungi, and oomycetes, and that such identification will increase our understanding of the interaction and co-evolution between pathogens and plant hosts. These studies will elucidate the molecular mechanisms of plant defense responses and will ultimately lead to the development of effective tools for controlling disease in the field.

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