Plant Small RNAs: Big Players in Biotic Stress Responses

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Abstract

A myriad of small RNAs (18–25 nt in length) undergo heterogeneous modifications to inflect RNA stability and other complex physiological processes like stress responses, metabolism, immunity, and epigenetic inheritance of environmentally acquired traits. Such small RNAs include microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and tRNA-derived small RNAs (tsRNAs). Worldwide crop production and human health are affected when plants are attacked by pathogens and pests. Therefore, a large collection of genes get up- or down regulated to mediate the defense responses in plants against pathogens (bacteria, fungi, oomycetes, and viruses). Host endogenous small RNAs, thus, come into play to counter biotic stress where RNA silencing machinery is utilized to facilitate pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity. RNA interference (RNAi) pathways trigger gene silencing in interacting species from even different kingdoms (cross-kingdom RNAi). Diverse pathways are involved in regulating the defense mechanism including Dicer-like proteins (DCLs), double-stranded RNA (dsRNA) binding protein, RNA-dependent RNA polymerases (RDRs),

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RNA polymerase IV and V, small RNA methyltransferase HEN1, and Argonaute (AGO) proteins showcasing their functional specificities as well as verbosity. Transgenic plants are newly emerging players that help in solving the problem of pathogen attack in fields. In this chapter, the recent breakthrough on the function of sRNAs in response to biotic stress, mainly in plant-pathogen interaction, and its application in disease control is discussed.

Keywords
Biotic stress · Small RNA · Cross-kingdom RNAi · Argonaute · Gene silencing

8.1 Introduction

8.1.1 Zigzag Model

World population is increasing at a constant rate leading to agricultural land loss. This problem caters for diverse means to improve global food production. Another problem accounted for is the loss in crop productivity and grain quality due to bacteria, fungi, oomycetes, viruses, and insects. Therefore, it is required to unleash the biotic stress responses in plants and develop innovative tools using traditional and modern breeding approaches for crop protection against pathogens and pests (Bebber and Gurr 2015). On the contrary, pathogens have acquired the ability to counter such barriers to access nutrients and flourish inside plants thereby provoking their immune system. Nevertheless, plants have derived a defense mechanism to overcome pathogen infection by activating or suppressing a large array of genes (Jones and Dangl 2006). The "zigzag model" is proposed which explains in an easier way the different layers of innate immunity when plants are infected with pathogens (Jones-Rhoades et al. 2006). To avoid spreading infection by pathogen, the very first means of defense against them is pattern recognition receptors (PRRs). These receptors are cell surface-localized, transmembrane proteins and can detect conserved pathogenic patterns known as microbe−/pathogen−/host danger-associated molecular patterns (MAMPs or PAMPs or DAMPs) and hence shoot up the MAMP-/PAMP-triggered immunity (MTI/PTI) to limit the spread of pathogen (Jones and Dangl 2006). Flagellin peptide, elongation factor Tu protein (EF-Tu), and chitin are the best-studied MAMPs that form a major component of fungal cell walls and lipopolysaccharides (LPS). The perception of MAMPs relies on PRRs where FLS2 and EFR recognizing flagellin and EF-Tu possess to have a same structural construction formed by extracellular leucine-rich repeats (LRR) and a cytoplasmic kinase domain. On the contrary, CERK1, an *Arabidopsis* PRR, recognizes chitin containing three extracellular LysM domains and a cytoplasmic kinase domain. This recognition helps in inducing callose deposition, producing reactive oxygen species, accumulating salicylic acid (SA), and expressing pathogenesis-related (PR) genes (Yang and Huang 2014). Pathogens, on the other hand, have developed schemes to outpower MTI by sending effector proteins inside plant cells

that abolish early recognition and downstream signaling events of MTI, therefore, resulting in effector-triggered susceptibility (ETS) (Feng and Zhou 2012). But plants too have emerged to protect themselves from this infection by using their resistance (R) proteins that recognize the specific effectors and activate effector-triggered immunity (ETI). This immune response is more sturdy and speedy (Chisholm et al. 2006). There is another hypersensitive response (HR), which causes cell death at the site of infection to restrain the growth of the pathogen. The effector proteins that are produced are called Avr factors. In the latter case, the R proteins [nucleotide binding site (NBS) and an LRR domain] guard the Avr factors and detect their modification caused by the effector proteins (Mackey et al. 2002). MAP kinase gets activated when pathogen's molecules are perceived by PRRs or R proteins leading to a reprogramming in host's gene expression along with the activation of genes with antimicrobial function (PR, pathogenesis related) (Tsuda and Katagiri 2010).

The war of defense and counter-defense between pathogens and plants has resulted in distinct collection of pathogen effectors and resistance genes.

8.2 Role of RNA

Posttranscriptional modifications are found extensively in stable and structured RNAs (tRNA and rRNA, mRNAs, and an expanding catalog of small and large noncoding RNAs) (Li and Mason 2014). Recent discovery of reversible 6-methyladenosine (m^6A) modifications in mRNAs (Dominissini et al. 2012) as well as key enzymes for their dynamic regulation is observed. Other studies have documented pseudouridine (Li et al. 2015), 5-methylcytidine (m^5C) (Hussain et al. 2013), and most recently, 1-methyladenosine (m^1A) (Dominissini et al. 2016) in mRNAs. RNA modifications are also observed in small RNAs to perform various cellular functions that include development in plants, metabolic study, maintenance of genome integrity, immunity against pathogens, and abiotic stress responses. Regulation of gene expression is performed by small RNA in a sequence-specific manner either transcriptionally or posttranscriptionally (Chapman and Carrington 2007).

Eukaryotic organisms possess 20–40-nucleotide (nt)-long noncoding RNA molecules called small RNAs, and depending on their biogenesis and precursor structure, small RNAs are placed in two discrete groups: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Yang and Huang 2014).

8.2.1 MicroRNA

Small noncoding RNA generated from an imperfectly base-paired hairpin structure with 21–24 nt is called miRNA (Chen 2009). MicroRNAs (miRNAs) negatively regulate gene expression at the posttranscriptional level through mRNA degradation or translation repression (Iwakawa and Tomari 2013). Plant miRNAs are derived

from the distinct noncoding transcripts of miRNA genes which are transcribed by enzyme RNA polymerase II. The primary miRNAs (pri-miRNAs) form a secondary fold-back structure and thereupon get processed by the RNase III-type enzyme Dicer-Like1 (DCL1) to create the precursor miRNAs (pre-miRNAs) (Rogers and Chen 2013). The miRNA duplexes once formed from pre-miRNA are stabilized by 2°-O-methylation and catalyzed by Hua Enhancer 1 (Yang et al. 2006) and transported to the cytoplasm by HASTY (Bollman et al. 2003). The passenger strand of the miRNA duplexes is often removed by unwinding or cleavage (Kawamata and Tomari 2010), and the guide strand is maintained in the RNA-induced silencing complex (RISC) that defines target recognition. Plant miRNAs exert a considerable effect on gene expression and mediate the cleavage of target mRNAs with near-perfect complementarity (Voinnet 2009).

8.2.2 SiRNA

Small interfering RNAs (siRNAs) are formed from near-perfect complementarity long double-stranded RNAs (dsRNAs) and are generated either from antisense transcription or by the action of RNA-dependent RNA polymerases (RDRs) (Katiyar-Agarwal and Jin 2010). There are many subclasses of siRNA present in plants depending on origin and biogenesis: trans-acting siRNAs (ta-siRNAs), heterochromatic siRNAs (hc-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), and long siRNAs (lsiRNAs).

8.3 RNA Silencing

Communication taking place between organisms whether pathogenic, parasitic, or symbiotic mediates the transport of regulatory molecules across the cellular boundaries between the host and its interacting pathogens/pests/parasites or symbionts. This triggers gene silencing in trans in the non-related species, a mechanism called cross-kingdom or cross-organism RNAi (Knip et al. 2014).

RNA interference (RNAi) is a gene silencing event that regulates sequence-specific gene and gets induced by double-stranded RNA (dsRNA). This results in inhibition of translation or transcription. Gene regulation is initiated by sRNAs in hosts or pathogens by posttranscriptional gene silencing (PTGS) or transcriptional gene silencing (TGS). PTGS is induced by miRNAs and siRNAs through messenger RNA (mRNA) cleavage/degradation or translational inhibition with the help of an RNA-induced silencing complex (RISC), while TGS is induced by siRNAs and some specific miRNAs. TGS is responsible for DNA methylation, histone modification, or chromatin modification (Cui and Cao 2014). A number of pathways are involved in producing regulatory small RNAs using various conserved protein families like the RNA-dependent RNA polymerases (RDRs), the double-stranded RNA-binding proteins (DRBPs), the Dicer-like proteins (DCLs), the small RNA methyltransferase (HEN1), and the Argonaute (AGO) proteins. Plant sRNAs and

RNA interference (RNAi) pathway components are major regulatory players in providing immunity to plants against viruses, bacteria, fungi, oomycetes, and pests (Seo et al. 2013). Transposable element (TE) regions transcribe sRNAs in filamentous plant pathogens, and silencing this TE can help in fighting infection (Chang et al. 2012).

8.4 RNA Silencing Suppressors of Pathogens

8.4.1 Viral Suppressors of RNA Silencing

Many viruses cipher specific proteins to suppress the host antiviral silencing response and to cause infection in them. These viral suppressors of RNA silencing (VSRs) perform at three different levels, i.e., they can (a) inhibit generation of viRNAs, (b) inhibit loading of viRNAs in RISC by binding to the viRNA, and (c) inhibit components of RISC. Table 8.1 discusses the mode of action of VSRs in plants.

8.4.2 Bacteria-Encoded Suppressors of RNA Silencing

Bacterial pathogens too have developed similar silencing suppressors to combat antibacterial defense responses in plants as in viruses. Navarro et al. (2008) identified several *Pst* type III secretion effectors that enhance the disease susceptibility by suppressing host RNA silencing machinery. Effectors include AvrPtoB which represses transcription of miRNA genes and lowers the level of pri-miR393, AvrPto which interferes with miRNA precursor processing and downregulates mature miR393 level, and HopT1 which inhibits the action of the AGO1 protein in the RISC complex. Likewise, fungi and oomycetes too have developed RNAi suppressors to counteract host antipathogen RNA silencing mechanisms.

8.5 Host Endogenous Small RNAs in Plant-Microbe Interactions

When pathogen interacts with its host at first, it triggers the immunity response in plants known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). Now, bacteria too rectify PTI by secreting and injecting effector proteins into plant cells leading to PTI suppression. Finally, host plant releases resistance components such as resistance (R) proteins that can recognize effectors and elicit effector-triggered immunity (ETI) (Chisholm et al. 2006). 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 such interactions, host endogenous small RNAs play a pivotal role in counteracting these pathogens.

Table 8.1 Mode of action of viral silencing suppressors in plants

Suppressor	Source	Mode of action	Reference
AC4	Geminivirus	Competes with AGOs by binding to single-stranded siRNA and thereby preventing RISC assembly	Chellappan et al. (2005)
AC2	Begomovirus	Transcriptional activator. Induces expression of any gene, which might be a silencing suppressor	Trinks et al. (2005)
HcPro	Potyvirus	Mimics *hen1* mutations. viRNAs are oligo-uridylated and partially degraded due to lack of 2-*O*-methylation	Wu et al. (2010a, b)
		Interacts with a calmodulin-related protein, overexpression of which suppresses silencing	
		Amino acids 180, 205, and 396 of HcPro are critical for suppression of miRNA, ta-siRNA, and VIGS pathway but not for sense PTGS	
P6	Cauliflower	Is imported in the nucleus and binds to DRB4 protein. Suppresses RNA silencing	Haas et al. (2008)
	Mosaic virus	Pathway, possibly by inactivating DRB4, which is an essential component required for DCL4 action	
2b	Cucumber	Interacts physically with siRNA-loaded RISC and inhibits its slicing action	Goto et al. (2007)
	Mosaic virus	In vitro assays suggest that 2b binds to siRNAs to a lesser extent than to long dsRNAs	
		2b inhibits the production of RDR1-dependent viral siRNAs	
P0	Polerovirus	Promotes ubiquitin-dependent proteolysis of AGO1	Pazhouhandeh et al. (2006)
P69	Tymovirus	Inhibits viRNA amplification	Chen et al. (2004)
AL2	Curtovirus	Interacts with adenosine kinase, whose inhibition possibly prevents methylation of viral DNA	Wang et al. (2005)
p126	TMV	Encodes methyltransferase and helicase. Binds duplex siRNA and inhibits HEN1-dependent methylation and degradation	Blevins et al. (2006)
RNAse III	*Closteroviridae*	In vitro assays suggest that RNAse III suppresses siRNA silencing by cleaving 21-, 22-, and 24-bp siRNAs into 14-bp fragments	Cuellar et al. (2009)

8.5.1 Noncoding Small RNAs

Small noncoding RNAs (sncRNAs), discovered in eukaryotes, are 18–30-nt-long molecules which perform numerous functions such as gene expression control, defense against other parasitic nucleic acids, epigenetic modification, and heterochromatin regulation (van der Krol et al. 1990). There are ample functions and beneficial applications reported so far. Few of them are encompassing cell-to-cell signaling and communication in multicellular organisms (Mittelbrunn and Sanchez-Madrid 2012), trans-generational RNAi (Bond and Baulcombe 2014) and memorization (Rasmann et al. 2012), cell fate differentiation and vascular formation (Benkovics and Timmermans 2014), systemic antiviral immunity (Saleh et al. 2009), environmental RNAi (Zhuang and Hunter 2012), cancer prevention and diagnosis (Salido-Guadarrama et al. 2014), and intercellular immune activation (Robbins and Morelli 2014). MicroRNAs (miRNAs) and small interference RNAs (siRNAs) are the best-studied sncRNAs.

In response to different pathogen stressors, various targets and functions of sRNAs are summarized in Table 8.2.

Table 8.2 Response of sRNA to different pathogen stressors

Small RNA	Small RNA source	Host/pathogen	Target genes	Expression of genes upon infection	Roles in plant pathogen infection
miR159	Plant	*Arabidopsis*/ bacterium *P. syringae*	MYB33, MYB65, MYB101	UP	Regulates gibberellin and ABA signaling pathways
miR160	Plant	*Arabidopsis*/ bacterium *P. syringae*	ARF10, ARF16, ARF17	UP	Increases PAMP-induced callose deposition
	Plant	*M. esculenta*/ fungus *C. gloeosporioides*	ARF10	UP	Regulates plant auxin and enhances plant defense response
miR167	Plant	*Arabidopsis*/ bacterium *P. syringae*	ARF8 and ARF6	UP	Regulates auxin signaling pathways and enhances plant defense responses
miR390	Plant	*Arabidopsis*/ bacterium *P. syringae*	TAS3	DOWN	Triggers the accumulation of ta-siRNAs that regulate arf3 and arf4 for auxin signaling

(continued)

Table 8.2 (continued)

Small RNA	Small RNA source	Host/pathogen	Target genes	Expression of genes upon infection	Roles in plant pathogen infection
miR398	Plant	*O. sativa*/fungus *M. oryzae*	SOD2	UP	Overexpression of miR398 increases the accumulation of hydrogen peroxide and defense-related genes and decreases fungal growth
miR399	Plant	*Citrus*/ bacterium Ca. *L. asiaticus*	PHO2	UP	Contributes to HLB symptoms and phosphorus homeostatis and signaling
miR408	Plant	Wheat/fungus *Puccinia striiformis f.* sp. *tritici*	TACLP1, a type of plantacyanin protein	UP/DOWN	Negatively regulates wheat resistance to stripe rust
miR1885	Plant	*Brassica napus*/ virus TuMV	TIR-NBS-LRR	UP	Represses ETI
nat-SiRNAATGB2	Plant	*Arabidopsis*/ bacterium *P. syringae*	PPRL	UP	Contributes to plant immunity by suppressing a negative regulator of the RPS2 pathway
AtlsiRNA-1	Plant	*Arabidopsis*/ bacterium *P. syringae*	AtRAP	UP	Contributes to plant immunity by silencing a negative regulator

8.6 Components of the Small RNA Biogenesis Pathway Play an Important Role in Plant Defense

Many plant genomes possess multiple components such as DCLs, RDRs, and AGOs in the RNAi silencing machinery. *Arabidopsis* has four DCLs, six RDRs, and ten AGOs, many of which are involved in plant defense signaling pathway.

8.6.1 Dicer-Like Proteins and Their Associated Proteins

Four DCLs present in *Arabidopsis* process dsRNA or fold-back RNA precursors to generate siRNAs and miRNAs, respectively. The role of DCLs and their compensatory functions in the production of virus-derived small RNAs (viRNAs) is well

understood using single, double, or triple mutants of DCLs in genetic experiments. A loss of function mutation in both DCL4 and DCL2 is enough to cause viral susceptibility (+ssRNA) in plants (Diaz-Pendon et al. 2007).

Qu et al. (2008) observed that all four DCL proteins, key components of RNA silencing pathway, are involved in providing an antiviral defense in plants with functional hierarchy as (DCL4>DCL2>DCL3>DCL1) in processing viral RNAs into viRNAs (Deleris et al. 2006). Other important cofactors like small dsRNA-binding proteins (DRBs) of DCL proteins are known, but these do not show hierarchical redundancy as do DCLs (Curtin et al. 2008). DRB4 when interacting with DCL4 confers resistance against viruses (Qu et al. 2008). On the contrary, DCL2 and DCL3 do not need interaction with DRB for production of viRNAs (Curtin et al. 2008). Another protein HEN1 containing dsRNA binding domain plays an important role in viral resistance (Park et al. 2002). When mutation was done in *hen1* of *Arabidopsis*, hyper-susceptibility to cauliflower mosaic virus (CMV) was observed in the plant as compared to wild type suggesting that HEN1 contributes to resistance against the virus (Boutet et al. 2003). Along with the abovementioned, DCL proteins are also involved in the production of small RNAs thereby giving antibacterial immunity in plants. The *dcl1* mutant showed heightened susceptibility to *Pst* DC3000 *hrcC*$^{-}$, a nonpathogenic strain that can evoke PTI (Navarro et al. 2008). HYL1, the dsRNA-binding protein associated with DCL1, is also involved in bacterial infection resistance as the *hyl1* mutant was susceptible to *Pst (avrRpt2)*.

8.6.2 RNA-Dependent RNA Polymerases

Elaborated studies have stated RDRs to be induced by antiviral defense as well as in the presence of defense signaling compounds such as salicylic acid (SA) (Xie et al. 2001). It was observed that when the expression levels of RDR1 are lowered in transgenic antisense *Arabidopsis* plants, viral RNAs get piled up and susceptibility to TMV and potato virus X (PVX) infection is increased. NtRDR1 is also involved in fighting against potato virus Y (PVY) infection and its ortholog AtRDR1 transmits defense against tobamovirus and tobravirus because *Arabidopsis rdr1* mutant plants had enhanced levels of viral RNAs (Yu et al. 2003). A functional homolog of AtRDR6, NbRDR6, provides resistance against viruses (Qu et al. 2005) as down-regulation of NbRDR6 increased the susceptibility to many different viruses at high temperatures.

8.6.3 Argonautes

Silencing of target genes is activated by AGOs as these are associated with small RNAs and form RISC complexes (Hannon 2002). In *Arabidopsis*, 10 AGOs are found to take part in plant immunity. hc-siRNAs promote transcriptional gene silencing (TGS) by guiding RNA-directed DNA methylation (RdDM) and histone modification in plants (Vaistij et al. 2002). AGO4 is a leading nuclear RNAi effector

associated with hc-siRNAs or ra-siRNAs that allows DNA methylation (Li et al. 2008) which links DNA methylation and plant defense together. Using both cytosine and histone methyltransferases, *Arabidopsis* plants silence viral chromatin of cabbage leaf curl virus (CaLCuV) and beet curly top virus (BCTV) (Raja et al. 2008). Viral suppressors AL2 and L2 stop adenosine kinase (ADK) activity which otherwise generates S-adenosylmethionine (a methyltransferase cofactor). Therefore, plants infected with virus in the absence of L2 had hypermethylation of viral DNA, and to recover from viral infection, AGO4 is needed (Raja et al. 2008). AGO4 also helps in antibacterial defenses. In addition to AGO4, AGO1 and AGO7 play a pivotal role in slicing viral RNAs (Qu et al. 2008). AGO1 is the primary slicer because it targets viral RNAs with more compact structures, but AGO7 is an alternate slicer which targets RNAs with less complexity. The biogenesis of AtlsiRNA-1 involved AGO7, as *ago7* mutant that does not accumulate AtlsiRNA-1 (Katiyar-Agarwal et al. 2006). However, other *ago* mutant plants, including *ago3*, *ago4*, and *ago9*, showed no significant change in the level of AtlsiRNA-1 as compared with wild type. AGO7 is also associated with TAS3 ta-siRNA (Fahlgren et al. 2006). AGO7 accumulates bacteria-induced AtlsiRNA-1 hence suggesting its role in antibacterial defense.

8.7 Cross-Kingdom RNAi and sRNA Trafficking

When two unrelated interacting organisms communicate with each other, it is called cross-kingdom RNAi. This process is observed in both animal and plant systems. Plants transfer RNAi signals into interacting organisms, such as filamentous fungi, oomycetes, nematodes, parasitic plants, and pests, to restrain their growth. This process is known as HIGS, the most noticeable example of cross-kingdom RNAi in plants (Koch et al. 2013). In order to develop pest- and pathogen-resistant crops, scientists have engineered diverse plant species, from model plants to commercial crops, so as to express exogenous artificial RNAi signals that suppress the gene of parasitic nematodes, herbivores, and fungal and oomycete pathogens by targeting their mRNAs (Koch and Kogel 2014). HIGS is functional and successfully used against parasitic plants such as *Orobanche* and *Cuscuta* spp. and in model plants such as *Arabidopsis thaliana* and tobacco *Nicotiana benthamiana* as well as in important crops, including wheat, barley, *Medicago*, and banana, to efficiently work against a variety of fungal and oomycete pathogens, such as *Blumeria graminis*, *Puccinia tritici*, *Fusarium* spp., and *Phytophthora capsici* (Koch and Kogel 2014). Basic mechanism of HIGS is that it alters the fungal morphology and growth inhibition in plants, thereby reducing virulence. Additionally, HIGS is also used to study gene function in non-transformable species (Yin et al. 2014). A HIGS approach was carried out on *Glomus* spp. to study gene function of the monosaccharide transporter 2 (Helber et al. 2011), showing that HIGS is functional on arbuscular mycorrhiza, which forms symbiotic relationship with hosts. Successfully applying HIGS helps plants to deliver mobile gene silencing signals for communication and manipulating diverse interacting organisms.

There are evidences of RNAi signaling taking place in the opposite direction. Advanced pathogens and parasites use cross-kingdom RNAi to suppress host

immunity for infection (Weiberg et al. 2015). Three Bc-sRNAs in *Botrytis*-host interaction suppress *Arabidopsis* and tomato immunity genes in vivo (Mayoral et al. 2014). It is also estimated that sRNAs are also likely to be exchanged between the parasitic plant and its host, but the study still awaits the research output. Secretion and uptake of protein and other macromolecules participate in providing barrier against pathogens and parasites (Huckelhoven 2007) and in pathogenesis and effector-triggered suppression of host plant immunity (Kale and Tyler 2011).

8.8 Small RNA Biogenesis Pathways in Plants

Arabidopsis is taken as a model plant to study small RNA pathways in plants. Generative work involves both forward and reverse genetic screens to study the cellular proteins participating in biogenesis and function of miRNAs and siRNAs. A brief review of different kinds of small RNA pathways known in *Arabidopsis* is discussed below.

8.8.1 Biogenesis and Mechanism of miRNAs in Plants

The very first observation of microRNAs (miRNAs) took place in a nematode *Caenorhabditis elegans* (Lee et al. 1993). These are also known as short temporal RNAs (stRNAs) because they were expressed temporally in a mutant nematode. These endogenous noncoding small RNAs accelerate the growth, development, and survivability of plants. Transcription of miRNA gene is carried out by RNA polymerase II forming primary transcripts (pri-miRNAs) as a stem-loop structure of 1000-bp-long nucleotides (Chen 2005). Two processing steps are involved in the formation of mature miRNAs. The first step is carried out inside the nucleus where the microprocessor complex acts on pri-miRNAs to pre-miRNAs (precursor miRNAs) of 60–70 nt long. Two proteins, Drosha (169 kDa, RNAse III protein) and Pasha (dsRNA binding protein/DGCR8), constitute the microprocessor complex (Creelman and Mullet 1997). Two orthologs of Drosha and Pasha, namely, Dicer-like 1 (DCL-1) and Hyponastic Leaves 1 (HYL-1), are engaged in preliminary processing step of miRNA biogenesis pathway in plants (Schauer et al. 2002). To allow second processing step occuring in the cytoplasm, HASTY transport protein (ortholog of exportin-5) is required to transport pre-miRNAs from nucleus to cytoplasm. In subsequent step, ATP-dependent RNAse III protein (Dicer) converts hairpin dsRNA (pre-miRNA) into 21–24-nt-long mature miRNA-miRNA∗ duplex with 2-nt 3′ overhangs. This enzyme recognizes 2-nt 3′ overhangs and eliminates about ~21-nt sequence from its ends (Du et al. 2011). Out of two strands in miRNA duplex, one is called as antisense miRNA (miRNA) which has G:U base pairs, mismatches, and unpaired base pairs at its 5′ end, while the other strand is known as sense strand (miRNA∗). A complex is formed between Argonaute 1 (AGO1) protein and one strand of miRNA to guide miRNA to target its complementary mRNA sequence. The destiny of target mRNA depends on the degree of its complementarity with associated miRNA sequence. Complete

degradation occurs from near-perfect complementarity, while repression of protein translation occurs from partial complementarity. This miRNA biogenesis pathway is under the feedback regulation by two principal miRNAs, miR162 and miR168, causing cleavage of DCL1 mRNA and AGO1 mRNA (Zhang et al. 2011), respectively.

The ability of miRNAs in crop improvement can be well documented as transgenic plants harbor miRNAs under constitutive and inducible promoters that can specifically downregulate target genes of interest with limited non-autonomous effect.

8.8.2 siRNA

Antisense transcription or cellular RNA-dependent RNA polymerase (RDR) is used to derive siRNAs. In plants, there are four discrete siRNAs present: trans-acting siRNAs (ta-siRNAs), natural antisense transcripts (NATs)-derived siRNAs (nat-siRNAs), heterochromatic siRNAs (hc-siRNAs) or repeat-associated siRNAs (ra-siRNAs), and long siRNAs (lsiRNAs). For the initiation of ta-siRNA formation, RNA Pol II transcribes noncoding TAS genes where long primary transcript products upon cleavage by miRNAs and RNA-induced silencing complexes (RISCs) produce a 5′ fragment or a 3′ fragment which acts as a template for complementary strand synthesis, also coordinated by RDR6 and SGS3 (Vazquez 2006). DCL4 and DRB4 act consecutively on dsRNA molecule to form ta-siRNAs (Gasciolli et al. 2005). Intersecting regions of sense and antisense transcripts of *cis*-NATs give rise to nat-siRNAs. RNA interference is exploited in order to accomplish desirable traits in crops by operating the gene expression (Table 8.3). After the identification of the target genes, RNAi construct with hairpin cassette was created. Plant transformation and later screening and traits evaluation take place.

8.8.3 miRNA vs. siRNA

The most important regulators of gene expression are microRNAs (miRNAs) and short-interfering RNAs (Vazquez 2006) having size of 20–24 nt long. The difference between the two lies in precursor structures, pathway of biogenesis, and modes of action (Axtell 2013) (Table 8.4). Both are processed from long RNA precursors by Dicer-like ribonucleases (Bernstein et al. 2001) and regulate the target gene repression (Hammond et al. 2000).

8.8.4 Transposon-Associated sRNAs in Eukaryotic Plant

Eukaryotic pathogens are capable of silencing TEs by producing transposable element (TE)-associated sRNAs. The transcription of sRNA effectors in *Botrytis cinerea* takes place via TEs to suppress host immunity-related genes. In return, host plant resistance (*R*) genes get clustered in genomic loci embellished with TEs. TEs

Table 8.3 Traits improved by targeting the specific genes in plants

	Traits improvement Biotic stress	Targeted gene	Plant	Reference
Virus resistance	*Bean golden mosaic virus* (BGMV)	*AC1*	Bean	Bonfim et al. (2007)
	Barley yellow dwarf virus (BYDV)	*BYDV-PAV*	Barley	Wang et al. (2000)
	Rice dwarf virus (RDV)	*PNS12*	Rice	Shimizu et al. (2009)
	Turnip yellow mosaic virus (TYMV)	*P69*	Tobacco	Niu et al. (2006)
	Turnip mosaic virus (TuMV)	*HC-Pro*	Tobacco	Niu et al. (2006)
Insect resistance	*Helicoverpa armigera*	*CYPAE14*	Cotton	Mao et al. (2007)
	Corn rootworm	*V-ATPase A*	Maize	Baum et al. (2007)
Nematode resistance	*Meloidogyne incognita*	Splicing factor and integrase	Tobacco	Yadav et al. (2006)
	Meloidogyne incognita	*16D10*	*Arabidopsis*	Huang et al. (2006)
Bacterial resistance	*Xanthomonas citri* subsp. *citri* (Xcc)	*PDS* and *CalS1*	Lemon	Enrique et al. (2011)
	Agrobacterium tumefaciens	*iaaM* and *ipt*	*Arabidopsis*	Escobar et al. (2001), Dunoyer et al. (2006)
Fungal resistance	*Magnaporthe grisea Xanthomonas oryzae*	*OsSSI2*	Rice	Jiang et al. (2009)
	Magnaporthe grisea	*OsFAD7* and *OsFAD8*	Rice	Yara et al. (2007)
	Phytophthora infestans	*SYR1*	Potato	Eschen-Lippold et al. (2012)
	Blumeria graminis f. sp. *tritici*	*MLO*	Wheat	Riechen (2007)
Enhanced drought tolerance		Farnesyl transferase	Canola	Wang et al. (2009)
		C-kinase 1 (*RACK1*)	Rice	Li et al. (2009)
		OsDSG1	Rice	Park et al. (2010)
		OsDIS1	Rice	Wang et al. (2011a, b)

show epigenetic control of *R*-gene expression by *R*-gene sRNAs. Likewise, pathogen protein effector genes occur as clusters and scatter with TEs. In another example, protein effector gene-derived sRNAs in *Phytophthora* spp. control the expression levels of effector. For both pathogen protein effector genes and host plant *R* genes, sRNAs play the crucial regulators assisted with TE transposition. TEs are a core source of sRNA production where pathogens allow regulation of TEs and TE-associated protein effector gene expression by sRNAs, delivering sRNA effectors into host cells to change host defense gene expression. In plants, the advent of

Table 8.4 A comparison of the types of sncRNAs

	siRNA	miRNA	ta-siRNA	nat-siRNAs
Derived from	Invasive nucleic acids (virus, transgenes, heterochromatin)	Noncoding regions. Distinct genomic loci. Encoded by own genes	Noncoding regions	Antisense genes
Transcribed by	Depends on origin	RNA pol II	RNA pol II	RNA pol II
Processed by	DCL, RDR, SDE, NRPD	DCL1, HYL1, HEN1	RDR6/ SGS3, DCL, miRNAs	DCL1, HYL1
Targets transcripts in	Cis	Trans	Trans	Cis
Binds to	AGO1, AGO2	AGO1	AGO1, AGO7	AGO1

sRNAs upon infection epigenetically controls *R*-gene expression thereby activating defense genes. There are chances that plants may deliver their own RNA or protein molecules into pathogen cells. These events affect plant-pathogen interaction to provide host resistance, pathogen virulence, and host adaptation.

8.9 sncRNAs and Viruses: New Frontiers of Defense

For universal gene expression changes, current studies affirm the use of sncRNAs in plant-virus interactions. It has been proposed that plant miRNA expression that targets plant transcripts changes its response virus recognition affecting both viral replication and spreading. Numerous plant miRNAs after viral infection get either up- or downregulated (Pacheco et al. 2012). For example, when turnip mosaic virus infects *Brassica rapa*, miR1885 is induced in its response and targets a TIR-NBS-LRR (TNL) disease resistance gene (He et al. 2008).

8.10 Biotic Stress Resistance

Ample economic loss is posed by plant pathogens due to depletion in crop production. Therefore, several RNAi strategies are on the board to provide improvement in crop defense mechanisms against various biotic stresses (viruses, bacteria, fungi, nematodes, and insects).

8.10.1 Virus Resistance

Virus-induced gene silencing (VIGS) is an RNA-mediated PTGS mechanism that allows plants to protect themselves from foreign gene invasion (Ding 2010).

Pathogen-derived resistance (PDR) provides plants resistance against virus through genetic engineering (Simon-Mateo and García 2011). This PDR is either protein mediated where transgene encodes the protein or RNA mediated where transgene forms the transcript. To attain PDR, hairpin dsRNAs including small hairpin RNA (shRNA), self-complementary hpRNA, and intron-spliced hpRNA are produced in vivo using inverse repeat sequences from viral genomes. This approach was used successfully to anchor resistance in cassava plants against African cassava mosaic virus (ACMV) (Vanderschuren et al. 2009). Another means of providing resistance against viruses is targeting the coat protein (CP) gene through RNAi. This strategy was shown by Powell-Abel et al. (2006) in transgenic tobacco expressing the CP gene of tobacco mosaic virus (TMV) thus giving resistance to TMV. This method was further utilized to generate resistance against many different viruses such as potato resistant to potato virus Y (PVY) (Missiou et al. 2004), tobacco resistant to beet necrotic yellow vein virus (BNYVV) (Andika et al. 2005), *Cucumis melo* resistant to papaya ring spot virus type W (PRSV-W) (Krubphachaya et al. 2007), *N. benthamiana* resistant to cucumber green mottle mosaic virus (CGMMV) (Kamachi et al. 2007), and *N. benthamiana* and *Prunus domestica* resistant to plum pox virus (PPV) (Hily et al. 2007). RNA silencing approach is not restricted to RNA viruses alone but also seen in DNA viruses. For example, following infection with geminivirus *Vigna mungo* yellow mosaic virus (VMYMV), blackgram plant recovers back when inoculated with hpRNA construct containing the promoter sequence of VMYMV under the control of the 35S promoter (Pooggin et al. 2003). On the advent of infection by turnip mosaic virus (TuMV) in *Brassica rapa*, two miRNAs, bra-miR158 and bra-miR1885, were greatly upregulated (He et al. 2008), the condition only seen in this particular interaction.

8.10.2 Bacterial Resistance

Bacteria spread at a speedy rate and therefore it is tough to control diseases caused by them. Supression of two genes of *Agrobacterium tumefaciens* carried out by RNAi involved in crown gall tumor formation (iaaM and ipt) also helps in reducing the production of tumors in *Arabidopsis* (Dunoyer et al. 2006). This approach could be further spread out to other plants. Resistance to plants from bacterial disease is negatively regulated by fatty acids and their derivatives (Jiang et al. 2009). Multiple pathogens can be resisted in *Arabidopsis* and soybean plants by RNAi-mediated suppression of *SACPD* gene that encodes for fatty acid desaturase (Jiang et al. 2009). In *Arabidopsis*, miR393 is said to repress auxin signaling by negatively regulating the F-box auxin receptors like *TIR1*, hence restricting the infection by bacteria *Pseudomonas syringae* (Navarro et al. 2006). Thus, transgenic *Arabidopsis* plants where miR393 is overexpressed have enhanced bacterial resistance with some developmental alterations (Navarro et al. 2006). But two different miRNAs, miR398 (Jagadeeswaran et al. 2009) and miR825 (Fahlgren et al. 2007), are said to be downregulated by bacterial infections. miR398 expression targets coding for two Cu/Zn superoxide dismutases that are CSD1 and CSD2 were analyzed, and it was observed

that CSD1 was upregulated on the outburst of bacterial infection in accordance with the downregulation of miR398 under biotic stress (Jagadeeswaran et al. 2009).

MiR482/2118 family of miRNAs were shown to target a number of NBS-LRR mRNAs encoding disease resistance proteins in tomato (*Solanum lycopersicum*) and other members of Solanaceae (Shivaprasad et al. 2012). MiR482-mediated silencing of *R* genes gets affected by viral and bacterial invasion. These miRNAs are either upregulated or downregulated and affect gene expression by either suppressing negative regulators or inducing positive regulators of immune responses.

8.10.3 Fungal Resistance

Fungal resistance is regulated by posttranscriptional gene silencing (PTGS). In *Arabidopsis* RNA silencing mutants *sgs2*, *sgs3*, *ago7*, *dcl4*, *nrpd1a*, and *rdr2* displayed exhibited heightened susceptibility to *Verticillium* strains (Ellendorff et al. 2009). In another example, RNAi-mediated suppression of a rice gene *OsSSI2* embellished resistance to blast fungus *Magnaporthe grisea* and leaf blight bacterium *Xanthomonas oryzae* (Jiang et al. 2009) by suppressing two genes, namely, *OsFAD7* and *OsFAD8* (omega-3 fatty acid desaturases) (Yara et al. 2007). Similarly, RNAi-mediated targeting of genes for lignin production led to enhanced resistance in soybean against phytopathogen *Sclerotinia sclerotiorum* due to reduced lignin content (Peltier et al. 2009). However, in case of wheat, 24 miRNAs are known to get affected by the fungus *Blumeria graminis f.* sp. *tritici* (Bgt) which is causing the deadly disease of wheat powdery mildew (Xin et al. 2010). On the other hand, rice miRNA osa-miR7695 negatively regulates a natural resistance-associated macrophage protein 6 (OsNramp6) against the blast fungus *Magnaporthe oryzae*. To overcome this disease, overexpression of Osa-miR7696 was carried out (Campo et al. 2013).

8.11 Biotechnological Use of Mobile sRNAS in Plants

Plant defenses against pathogens and pests get accelerated by the discovery of sRNAs as mobile gene regulators thereby providing alluring and new strategies for crop improvement (Koch and Kogel 2014). HIGS, too, has played a great role in efficiently providing resistance against distinct plant herbivores, nematodes, and filamentous pathogens, when targeting important virulence genes. HIGS is a well-known tool under controlled lab conditions when applied to specific host and definite pathogen, but in field conditions, their suitability is compromised due to fluctuating environmental stresses and humungous variation in genes of pathogen and pest populations. Thus, more advanced studies and experimentation are needed to carry forward. Transportation of sRNA in different interactions such as plant-pathogen, plant-parasite, or plant-symbiont has made it feasible to construct the beneficial fungi or disarmed pathogens (with essential virulence genes deleted) and alter plant physiology via trans-kingdom gene silencing. Moreover, when the target

pathogen mRNAs are emphasized, a broad range of pathogens and pests can be controlled in a transgene-free plant framework via RNAi signals. RNA silencing-based technique can be further strengthened when a decent knowledge on molecular mechanisms of RNA communications and transport between plants and interacting organisms is attained. While genetically engineered crops have always been under domain of public eye, an understanding of cross-kingdom RNAi may help relieve public concerns. Some more applications of mobile sRNAs in plants are in metabolic engineering and systemic-induced resistance (Saurabh et al. 2014). Even food RNAi might become an important part of plant food-based technologies in the future (Hirschi 2012). Feeding studies stated that oral uptake of sRNA-containing nutrients led to accumulation of food-borne sRNAs in body fluids and organs, indicating their partial survival inside the intestinal tract (Liang et al. 2014). Research is ongoing to see if food-borne sRNAs have any negative or positive impacts on the physiology of the individual who consumes foods with plentiful sRNAs (Dickinson et al. 2013).

8.12 Conclusion

Research in sncRNAs is ultimately one of the most effective and encouraging fields in plant defense biology, and many more advances are waiting to be explored in this area of research. A large number of studies discussed here emphasize on the significance of sncRNAs in gene regulation in response of plants to pathogens (viruses, bacteria, and fungi). The induction and repression of sncRNAs in plants toward pathogens depend upon the incompatible and compatible interactions indicating that these RNAs can both act as positive and negative regulators of plant immunity. Biotechnological tools and strategies need to be implemented to speed up the resistance studies in plants against various pathogens. During symbiotic interactions, relevance of repression of R genes provides a bridge between pathogenic and beneficial interactions. When effectors interact with the plant silencing machinery, pathogens can surpass the plant immunity mechanisms. Since the complete annotation of sequence of miRNAs involved in biotic stresses still needs to be carried out in crop plants like rice, maize, soybean, mustard, *Jatropha*, barrelclover, etc., genes of small RNAs (miRNAs) can be used for analysis of stress tolerance in biotic conditions. Computational methods and high-throughput techniques like miRNA microarray, real-time PCR, or northern blot are utilized to identify expressed miRNAs and their target(s) which provide plant defense against various biotic stresses. Studying the complexity of regulation these proteins had to undergo in order to provide crops resistance against pathogens is required. Comparing the antiviral and the antibacterial roles of the small RNA biogenesis factors may shed light on the complex modes of regulation these proteins have to undergo to confer plants' disease resistance. The study of VSRs and BSRs along with their targets may help to solve redundancy in the activity of several RNA silencing components during plant-microbe interactions. An insight into plant defense mechanisms will help to improvise crops of economic importance which should be pathogen-free too.

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