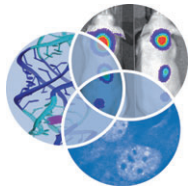
Advanced Review



Small RNAs: essential regulators of gene expression and defenses against environmental stresses in plants

Hsiao-Lin V. Wang and Julia A. Chekanova*

Eukaryotic genomes produce thousands of diverse small RNAs (smRNAs), which play vital roles in regulating gene expression in all conditions, including in survival of biotic and abiotic environmental stresses. SmRNA pathways intersect with most of the pathways regulating different steps in the life of a messenger RNA (mRNA), starting from transcription and ending at mRNA decay. SmRNAs function in both nuclear and cytoplasmic compartments; the regulation of mRNA stability and translation in the cytoplasm and the epigenetic regulation of gene expression in the nucleus are the main and best-known modes of smRNA action. However, recent evidence from animal systems indicates that smRNAs and RNA interference (RNAi) also participate in the regulation of alternative pre-mRNA splicing, one of the most crucial steps in the fast, efficient global reprogramming of gene expression required for survival under stress. Emerging evidence from bioinformatics studies indicates that a specific class of plant smRNAs, induced by various abiotic stresses, the sutr-siRNAs, has the potential to target regulatory regions within introns and thus may act in the regulation of splicing in response to stresses. This review summarizes the major types of plant smRNAs in the context of their mechanisms of action and also provides examples of their involvement in regulation of gene expression in response to environmental cues and developmental stresses. In addition, we describe current advances in our understanding of how smRNAs function in the regulation of pre-mRNA splicing.

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INTRODUCTION

Small RNAs (SmRNAs) are indispensable in regulating eukaryotic gene expression during various stages of organism's development and under different conditions, including survival of environmental stresses that perturb the state of a cell or organism. To survive these perturbations, plants acclimate to stresses by fine-tuning gene expression in response to environmental conditions. These changes in gene expression produce both general and stress-specific molecular responses that help the plant to acclimate to the changes in the environment (Figure 1(a)). As in other eukaryotes, regulation of gene expression in plants relies on a variety of molecular mechanisms that affect different steps in the life of a messenger RNA (mRNA), including transcription, splicing, processing, transport from the nucleus to the cytoplasm, translation, storage and mRNA decay (Figure 1(a)). By regulating gene expression mainly through transcriptional and post-transcriptional gene silencing (PTGS), smRNAs function as major players controlling different steps of mRNA life (Figure 1(b), bottom). Moreover, emerging evidence in animal

*Correspondence to: chekanovaj@gmail.com

School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO, USA

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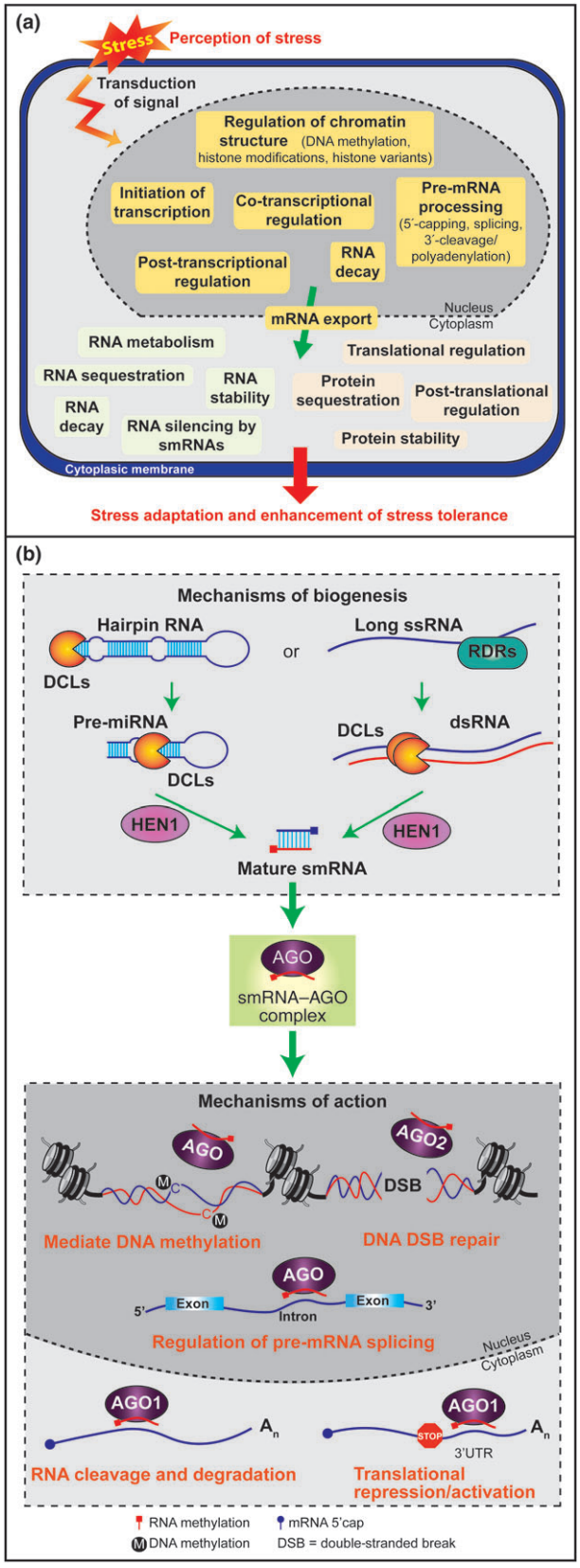

systems indicates that smRNAs participate in the regulation of pre-mRNA splicing, which can trigger global changes in transcriptomes and reprogramming of gene expression and thus provide the rapid responses vital for survival of stresses.

Eukaryotes have at least three major classes of smRNAs: microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs); miRNAs and siRNAs occur widely in plants and animals, but piRNAs are found only in animals. Most plant smRNAs are ~20–24 nucleotides long and undergo complex biogenesis.[2] In general, miRNAs originate from *MIR* genes transcribed by RNA polymerase II (Pol II) and derive from distinctive double-stranded RNA (dsRNA) precursors that can form hairpin structures[3,4] (Figures 1(b), top and 3 (a)). SiRNAs can originate from dsRNA precursors that form by various mechanisms, most often (in plants) by RNA-directed RNA polymerases (RDRs). Dicer proteins (RNase III domain-containing endoribonucleases), which in plants are called Dicer-like (DCL) proteins, process the miRNAs and siRNAs from these dsRNA precursors (Figure 1(b), top). Plant smRNAs, as well as piRNAs and some animal siRNAs, are 2′-O-methylated on the 3′ terminal ribose by the methyltransferase HEN1 to increase their stability.[5] In contrast to other systems, plant siRNAs are very diverse and include several subclasses, based on their origin and biogenesis (Table 1).

Mature smRNAs are incorporated into Argonaute (AGO) complexes and function as sequence-

FIGURE 1 | SmRNAs in the regulation of gene expression in response to stresses. (a) Environmental stresses trigger rapid changes in gene expression. Adaption to environmental stresses requires rapid changes in gene expression on all levels in both nuclear and cytoplasmic compartments. These coordinated events result in global reprogramming and enhancement of stress tolerance. (b) Biogenesis and functions of smRNAs. SmRNAs are produced from dsRNA precursors. MiRNAs derive from the stems of hairpin-forming *miRNA*s (top left), while siRNAs are produced from dsRNA precursors, which can be formed in various ways, most often by RNA-directed RNA polymerases (RDRs) in plants (top right). Dicer proteins process the dsRNAs into mature smRNAs that are methylated at their 3′ end by the methyltransferase HEN1 to increase their stability and then incorporated into AGO effector complexes. The smRNA–AGO complexes regulate gene expression on multiple levels (bottom box). In the nucleus, smRNAs can mediate DNA methylation and histone modifications, participate in DNA double-stranded break repairs, or regulate pre-mRNA splicing. In the cytoplasm, smRNAs regulate mRNA decay and act in the regulation of translation. The products of smRNA-mediated cleavage are generally subjected to the 3′→5′ exonucleolytic degradation by the exosome and 5′→3′ degradation by the exoribonuclease Xrn4 (the *Arabidopsis* homolog of yeast Xrn1).[1]

TABLE 1 | Main Classes of *Arabidopsis* smRNAs

Name	Length (nt)	Origin	Functions	References
miRNA	~20–22	DsRNAs with hairpin-shaped secondary structure at miRNA loci	Mediate gene silencing post-transcriptionally by mRNA cleavage or regulation of translation via perfect or near-perfect complementarity with target RNAs	Extensively reviewed in Refs 3,6
lmiRNA	~23–27	DsRNAs with hairpin-shaped secondary structure at miRNA loci	Mediate DNA methylation in *cis* and *trans*, leading to transcriptional gene silencing	7–9
het-siRNA (or hc-siRNA)	24	Transcripts from heterochromatin, transposons, and repetitive sequences	Function in heterochromatin formation and RdDM pathway; involved in establishing and maintaining DNA methylation and histone modifications	2,10,11
tasiRNA (phasiRNA)	21	MiRNA-triggered phased processing of *TAS* transcripts	Mediate post-transcriptional gene silencing, acting as morphogens by controlling expression gradients of the target mRNAs; can also mediate DNA methylation in *cis*	Refs 12–18, and extensively reviewed in Ref 19
nat-siRNA	20–22 or 23–26	*Cis*- or *trans*-NATs induced by stresses	Mediate gene silencing mainly at the post-transcriptional level by mRNA cleavage in stress conditions or developmentally	Refs 20–22, and extensively reviewed in Ref 23
lsiRNA	30–40	Single protein-coding regions or NATs	Induced by pathogen infection and downregulate mRNA expression by promoting mRNA decapping and 5′→3′ degradation	24
diRNA	21 and 24	Produced in the vicinity of DNA DSB sites	May function as guide molecules directing the recruitment of protein complexes to DSB sites to facilitate repair	25
easiRNA	21	Reactivated transposons during reprogramming in vegetative nucleus in male germ line	Mediate transcriptional silencing of transposons in epigenetically reprogrammed male germ line	26,27
Sutr-siRNA[1]	24	3′ UTRs of coding genes in response to abiotic stresses	Proposed to mask or affect accessibility of specific intronic regulatory *cis*-elements via base-pairing to promote the choice of correct splice sites during environmental stresses	28

miRNA, microRNA; lmiRNA, long microRNA; het-siRNA, heterochromatic siRNA; tasiRNA, *trans*-acting siRNA; nat-siRNA, natural antisense transcript-derived siRNA; lsiRNA, long siRNA; diRNA, double-stranded break-induced siRNA; easiRNA, epigenetically activated siRNA; sutr-siRNA, stress-induced UTR-derived siRNA; dsRNA, double-stranded RNA; NATs, natural antisense transcripts; DSB, DNA double-stranded breaks.

[1] Have been identified in *Brachypodium distachyon*.

specific guides for inactivating homologous sequences via degradation of mRNAs, inhibition of translation, or repression of transcription, a set of mechanisms collectively known as RNA interference (RNAi).[4,10,29] The miRNA duplex assembles with AGO proteins into the RNA-induced silencing complex (RISC), which then base pairs with mRNAs and induces smRNA-directed PTGS through mRNA degradation and/or translational repression in the cytoplasm[3,4] (Figure 1(b), bottom). In addition to their roles in the cytoplasm, plant miRNAs can also function in the nucleus to trigger the production of phased, secondary siRNAs, such as the extensively mobile *trans*-acting siRNAs (tasiRNAs) as well as phasiRNAs.[19,30] Emerging evidence indicates that in some cases miRNAs can also participate in transcriptional gene silencing (TGS) by mediating DNA methylation.[7]

Plant siRNAs affect translation and cleavage of target RNAs similarly to miRNAs, but they are the primary group of plant smRNAs responsible for eliciting TGS by directing repressive epigenetic modifications, such as DNA cytosine methylation and/or histone methylation, to homologous regions of the genome.[11,31] This group of heterochromatic plant siRNAs functions similarly to the nuclear siRNAs functioning in co-transcriptional gene silencing in *Schizosaccharomyces pombe*.[29,32] SiRNAs also participate in DNA double-stranded break repair in plants and humans[25,33] (Figure 1(b), bottom).

Examples of smRNAs regulating gene expression are largely limited to their effects on epigenetic regulation, translation, and mRNA stability, but emerging evidence also indicates that RNAi and smRNAs can affect pre-mRNA splicing. Splicing functions as a powerful regulator of gene expression and most eukaryotic genes also undergo alternative splicing (AS), in which different exons are selected in a pre-mRNA transcript to produce different mRNAs.[34–36] Various connections between RNAi, chromatin, and splicing have recently emerged and work in animals showed that the RNAi machinery regulates AS by affecting the elongation rate of RNA Pol II, providing a connection between epigenetic mechanisms and the splicing machinery.[37–42] The regulation of splicing in response to environmental stresses is particularly important, as survival under stress requires rapid and dramatic reprogramming of gene expression and is accompanied by profound changes in global patterns of pre-mRNA splicing.[43] A recent report describing a group of stress-induced sutr-siRNAs with complementarity to specific *cis*-elements important for splice-site selection in *Brachypodium* suggests that smRNAs may potentially function in the regulation of splicing by masking or changing accessibility of specific *cis*-elements to mediate gene expression in response to stresses.[28]

In the following sections, we explore the different functions of plant smRNAs, focusing on their mechanisms of action. We begin with the effects of siRNAs on epigenetic regulation and TGS, including RNA-directed DNA methylation, and then examine the roles of smRNAs, including miRNAs, tasiRNAs, natural antisense siRNAs (nat-siRNAs), and long siRNAs (lsiRNAs), in PTGS, including the post-transcriptional degradation of mRNAs. After examining these well-known functions of smRNAs, we next explore two less well-known functions of smRNAs, in the repair of DNA double-strand breaks (DSBs) and in the regulation of pre-mRNA splicing.

Within each section, we first describe the mechanisms to provide context, then the types of plant smRNAs that predominantly act in these mechanisms, and finally provide examples of the described mechanisms in response to stress. As plants have an incredibly rich variety of smRNAs and broad range of RNAi pathways, we can only provide a quick sketch of each pathway, with a focus on the role of smRNAs in the regulation of splicing. We thus direct readers to specialized reviews that describe these pathways and for added information on RNAi, stress responses, and regulation of splicing.

EPIGENETIC SILENCING VIA siRNAs AND DNA METHYLATION

The most prominent role of plant siRNAs is their function in eliciting TGS by directing repressive epigenetic modifications to homologous regions of the genome.[11,31] In this section, we examine the roles of heterochromatic siRNAs (het-siRNAs) in RNA-directed DNA methylation (RdDM) and epigenetically activated siRNAs (easiRNAs) in maintaining genomic stability, via repression of transposable element (TE) activation in germ cells, and provide examples of how these smRNAs may help plants defend against stresses.

Het-siRNAs and the RdDM Pathway

Het-siRNAs are derived mostly from silenced repetitive sequences and TEs and are recruited back to the source or homologous chromatin to trigger DNA methylation and histone modifications resulting in transcriptional silencing. This process in plants, termed RdDM, has remarkable mechanistic parallels to co-transcriptional gene silencing in *S. pombe*.[11,29]

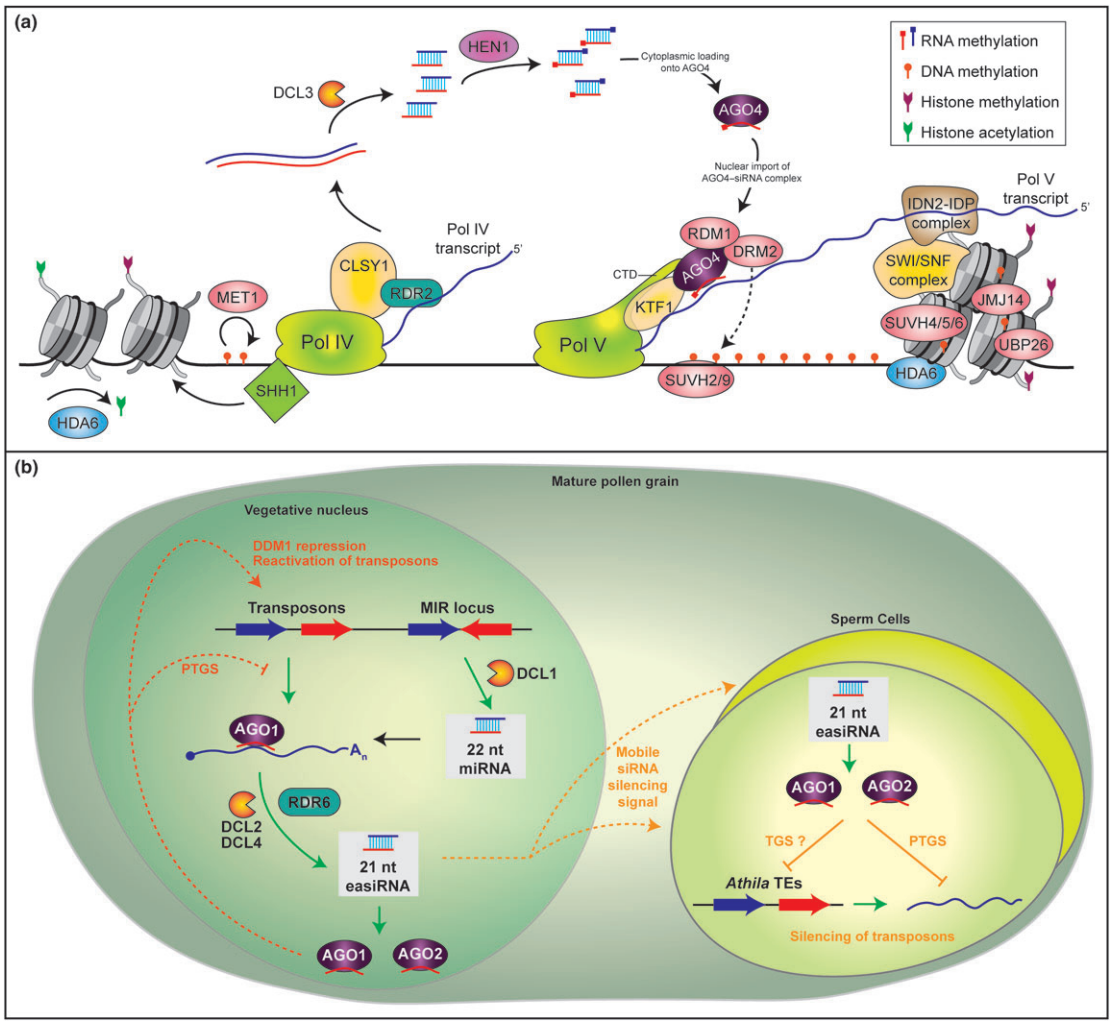

FIGURE 2 | SmRNAs in transcriptional gene silencing (TGS). (a) The RNA-dependent DNA methylation pathway (RdDM) and het-siRNAs. Pol IV transcripts serve as precursors for het-siRNAs, while Pol V-produced lncRNA scaffolds act as targets recognized by siRNAs. Pol IV is recruited to its genomic loci by SHH1 (H3K9me reader) and the SNF2 domain-containing chromatin remodeler CLSY1 facilitates Pol IV transcription.[44] RDR2 converts single-stranded Pol IV transcripts into dsRNA and these dsRNAs are further processed by DCL3 into mature 24-nt het-siRNAs. Mature het-siRNAs are stabilized by methylation at the 3′ end by HEN1 and exported to the cytoplasm and loaded onto AGO4. AGO4–siRNA complexes are reimported into the nucleus to guide the targeting of nascent Pol V scaffold transcripts by sequence complementarity. Pol V transcription is facilitated by the DDR complex[45] and SUVH2 and/or SUVH9 (H3K9 methyltransferase) aid Pol V recruitment to its genomic loci.[44] The IDN2–IDP complex bound to Pol V scaffold RNAs interacts with SWI/SNF complex, which adjusts nucleosome positioning.[46] The interaction of AGO4 and KTF1 (a putative transcription elongation factor) aids in recruiting AGO4–siRNA complexes to Pol V transcripts; the AGO4–siRNA complex pairs with Pol V transcripts and, along with RDM1 (RNA-DIRECTED DNA METHYLATION 1), recruits DRM2 (cytosine-5-methyltransferase), which catalyzes de novo cytosine methylation to silence the locus. There is crosstalk between the DNA and H3K9 methylation pathways. H3K9 methylation by KYP (SUVH9), SUVH5, and SUVH6 amplifies silencing mediated by DNA methylation (extensively reviewed in Ref 11). Together this results in transcriptional silencing at the genomic loci that are transcribed by Pol IV and Pol V, particularly TEs and other repetitive DNA. Adapted from Refs 11, 47, 48. (b) EasiRNAs. EasiRNAs are functionally equivalent to animal-specific piRNAs. Developmental stresses during the reprogramming of the plant germ line trigger easiRNA production. Unlike animals, plant germ cells arise from somatic stem cells. The *Arabidopsis* male gametophyte (pollen grain) is binucleate, with one large vegetative cell enclosing a smaller cell that eventually gives rise to two sperm cells. The vegetative nucleus (VN) performs only supportive functions and does not contribute DNA to the next generation. Reprogramming in the germ line coincides with loss of chromatin remodelers in the VN. In the VN, *DDM1* expression is repressed, leading to reversible chromatin decondensation and DNA demethylation of transposons, which reactivates TE transcription and triggers easiRNA production via a pathway requiring RDR6, DCL4/DCL2, and AGO1/AGO2. Over 50 known endogenous miRNAs, which target TEs post-transcriptionally, can also trigger production of easiRNAs. The 21-nt easiRNAs from the VN can move to induce PTGS in the nuclei of the sperm cells. They may also inhibit epigenetic modification to affect TEs. The easiRNAs can also form via an miRNA-independent pathway, but the details of this pathway remain to be elucidated. Adapted from Refs 10, 26, 27.

RdDM requires two plant-specific RNA polymerases, Pol IV and Pol V, with some involvement of RNA Pol II in establishing DNA methylation and histone modifications. By contrast, in *S. pombe*, RNA Pol II fulfills the roles of Pol IV and Pol V, resulting in H3K9 methylation (see RdDM description in Figure 2(a)).[11] The majority of 24-nt het-siRNAs are derived from Pol IV-produced lncRNAs, which are made double-stranded by RDR2 and processed by DCL3.[2,50] The het-siRNAs incorporated into AGO4 recognize lncRNA scaffolds produced by Pol V via sequence complementarity and, together with other factors, recruit DNA methyltransferase to mediate *de novo* cytosine methylation, resulting in transcriptional silencing of the locus.

Recent work also identified a noncanonical RDR6-RdDM pathway, which is independent of Pol IV and DCL3.[11,49] In noncanonical RDR6-RdDM, RDR6 copies Pol II-derived transcripts dsRNAs, which DCL2 and DCL4 then process into 21–22-nt siRNAs.[49] If loaded onto AGO1, these noncanonical siRNAs can induce PTGS; if loaded onto AGO2, they can initiate *de novo* DNA methylation, triggering RdDM. In *Arabidopsis,* Pol IV generates most siRNAs, although siRNA biogenesis in plants is complex and Pol V (and to a lesser extent Pol II) can also produce the templates for siRNAs (for a review of RdDM pathways, see Ref 11).[50–53]

Various environmental stresses, including temperature, UV, drought, salinity, and pathogen infections, can affect epigenetic regulation leading to transcriptional activation of many silenced loci, such as silent transgenes, TEs, and loci within constitutive heterochromatin.[54–57] In some cases, stress-induced activation of silenced loci can occur without changes in DNA methylation or repressive histone marks, but stresses usually trigger destabilization of chromatin states, manifested as changes in DNA methylation and reductions in nucleosome occupancy.[58] Although stress can induce long-term changes in epigenetic marks and generate epialleles that could be stably transmitted,[59] stress-induced chromatin changes are usually carefully guarded from *trans*-generational transmission by active epigenetic regulation. For example, preventing *trans*-generational transposition of the *ONSEN* transposon, which is activated in response to heat stress, requires components of the RdDM pathway, Pol IV, NRPD2 (the common subunit of RNA Pol IV and Pol V), RDR2, DCL3, and SUVH2 (a putative H3K9 methyltransferase).[60] The nucleosome remodeler DDM and the TGS regulator MOM1 also participate in preventing stress-induced epigenetic changes from being transmitted in *Arabidopsis*.[61]

Acclimation to stresses in *Arabidopsis* requires epigenetic pathways. For example in *Arabidopsis*, tolerance to salt and freezing stress requires histone deacetylase complexes.[62–64] The RdDM pathway also acts in basal heat tolerance in *Arabidopsis*; for example, *hda6* (Rpd3-type histone deacetylase HDA6) mutants and RdDM pathway Pol IV/Pol V mutants are hypersensitive to heat stress. The mutants of other components of RdDM, such as RDR2, DCL3, and AGO4, also show a moderate reduction in survival rates.[55] In the heat stress response in *Arabidopsis*, the Pol IV/V and HDA6 pathways function independently at different steps. However, the genes that show altered transcription in Pol IV/Pol V mutants under heat stress neighbor TE remnants or regions that produce siRNAs, suggesting that the altered heat responsiveness of protein-coding genes results from defective epigenetic regulation of nearby TEs in plants deficient in RdDM. Epigenetic pathways also act in response to stress in organisms other than plants; for example, in *S. pombe*, nuclear Dicer (Dcr1) directly regulates heat-stress-responsive genes via co-transcriptional gene silencing.[65] In response to heat stress in *S. pombe* cells, Dcr1 moves from the nucleus, resulting in activation of stress-related genes.

In addition to acting in abiotic stresses, RdDM also participates in plant responses to infection by bacterial pathogens. Infection with *Pseudomonas syringae* triggers widespread, dynamic changes in DNA methylation in the plant genome via both downregulation of RdDM and demethylation; these changes lead to increased levels of TE transcripts, synthesis of siRNAs, and often transcriptional changes in neighboring genes.[58,66] Also, during tumor formation by pathogenic *Agrobacterium*, the host genome undergoes hypermethylation. *Arabidopsis* mutants deficient in RdDM components exhibit altered resistance to bacterial pathogens;[58,66–68] for example, *ago4* mutants and *cmt3–drm1–drm2* (DNA methyltransferases) triple mutants have enhanced tumor development, suggesting that in this example hypermethylation could be a defense mechanism driven by host RdDM.[68] Pathogen-triggered changes in host methylation indicate that both hosts and pathogens might alter RdDM, but such changes may be a mechanism of pathogenesis or a part of the host defense response.

Epigenetically Activated siRNAs

Developmental stress associated with erasure of epigenetic marks during the reprogramming of the germ line in plants involves *Arabidopsis* 21–22-nt

easiRNAs, which specifically target TE transcripts to prevent transposon activation during reprogramming. EasiRNAs functionally resemble the PIWI-interacting piRNAs in animal germ lines and accumulate in the vegetative nucleus of the pollen grain due to the loss of heterochromatin, which leads to TE activation. They also accumulate in *met1* (DNA methyltransferase) and *ddm1* (nucleosome remodeler required for DNA methylation of TEs) mutants[26] (Figure 2(b)).

The 24-nt transposon silencing het-siRNAs (described above) form directly from dsRNA precursors, but some easiRNAs are produced atypically and genetically resemble 21-nt tasiRNAs and other secondary siRNAs (discussed below), which are triggered by miRNAs. Endogenous miRNAs target the transcripts of the reactivated transposons and RDR6 forms dsRNA from the resulting fragments.[27] EasiRNAs are processed by DCL4 and DCL2, and incorporated into AGO1 and AGO2.[49,69,70] EasiRNAs can also regulate gene expression and the response to environmental stress. For example, siRNA854, which is highly conserved from plants to mammals (known as miRNA854 in animals), regulates the *UBP1b* mRNA, which encodes TIA-1, a protein required for the formation of stress granules.[71,72] The *ubp1b* mutants cannot form stress granules and display increased sensitivity to stress.[69]

SMALL RNA-MEDIATED POST-TRANSCRIPTIONAL DEGRADATION OF mRNAs

The mechanism of smRNA-mediated PTGS is well conserved across eukaryotes and includes translational inhibition and degradation of cytoplasmic mRNAs. Degradation of mRNAs by RISC is the classic method by which smRNAs post-transcriptionally inhibit gene expression.

Here, we briefly examine the role of miRNAs (and long miRNAs) in mRNA degradation and provide examples of how they help plants defend against environmental challenges. Finally, we examine secondary, tasiRNAs and nat-siRNAs. For brevity, we omit in-depth discussion of conventional miRNA biogenesis and functions; the roles of smRNAs in stress responses in plants have also been extensively reviewed. The functions of smRNAs in virus-induced gene silencing (VIGS)[73,74] and the role of miRNAs in translational repression[3,4] are not described here and thus we also direct the reader to recent, excellent reviews on these topics.

MiRNAs and Long miRNAs

Canonical plant miRNAs are 20–22-nt long, require DCL1 for their biogenesis, and function in complex with AGO1, although exceptions have been described for each of these generalizations.[3,30] Plant miRNAs exhibit extensive complementarity to their mRNA targets, unlike animal miRNAs, which rarely have perfect complementarity to their targets and rely on the 'seed region' (2–7 or 8 nt from the 5′ end), which carries the key sequences required for recognition of target mRNAs.[75,76] Most plant miRNAs regulate gene expression at the post-transcriptional level (Figure 3(a)). Variation in the length of miRNAs may also define their distinct functions. For example, 22-nt miRNAs have an additional function in triggering production of secondary tasiRNAs.[12] Another class of plant miRNAs is 23–27-nt long, termed long miRNAs (lmiRNAs); these can be produced from the same *MIR* gene that also gives rise to canonical 20–22-nt miRNAs.[8] In contrast to canonical miRNAs, which are generated by DCL1 and associate with AGO1, lmiRNAs arise as a result of competition between DCL3 and DCL1 in processing of the same double-stranded stem-loop precursors and associate with AGO4[9] (Figure 3(a)). The accumulation of lmiRNAs also requires RDR2 and Pol IV, components of typical heterochromatin siRNAs biogenesis. Studies in *Arabidopsis* and rice indicate that AGO4–lmiRNA complexes are functionally distinct from AGO1–miRNA complexes. AGO4–lmiRNA complexes are functionally similar to het-siRNAs and can direct DNA methylation in *cis* at loci where they are produced and also in *trans* at their targeted loci.[7,9]

A number of stress-regulated miRNAs have been identified in plants under various biotic and abiotic stress conditions and many plant miRNAs play important roles in adaptations to stress.[6] The functions of miRNAs in stress responses in plants and stress signaling pathways have been extensively summarized in recent reviews.[77,78] For example, *MIR168a* is specifically transcriptionally induced by abiotic stress or by treatment with abscisic acid (ABA), the plant stress hormone, and miR168 binds to ABA-responsive *cis* element within the *MIR168a* promoter.[79] MiR168a mediates a regulatory feedback loop by controlling homeostasis of its target, *AGO1* mRNA, during stress responses and is required for ABA and drought tolerance.

MiRNAs also participate in the defense against pathogenic attacks.[73] Various pathogens can produce the plant hormone auxin or modulate the host's auxin levels to facilitate infection. *Arabidopsis* miR393 is induced upon recognition of bacterial infection and alters plant immunity by suppressing

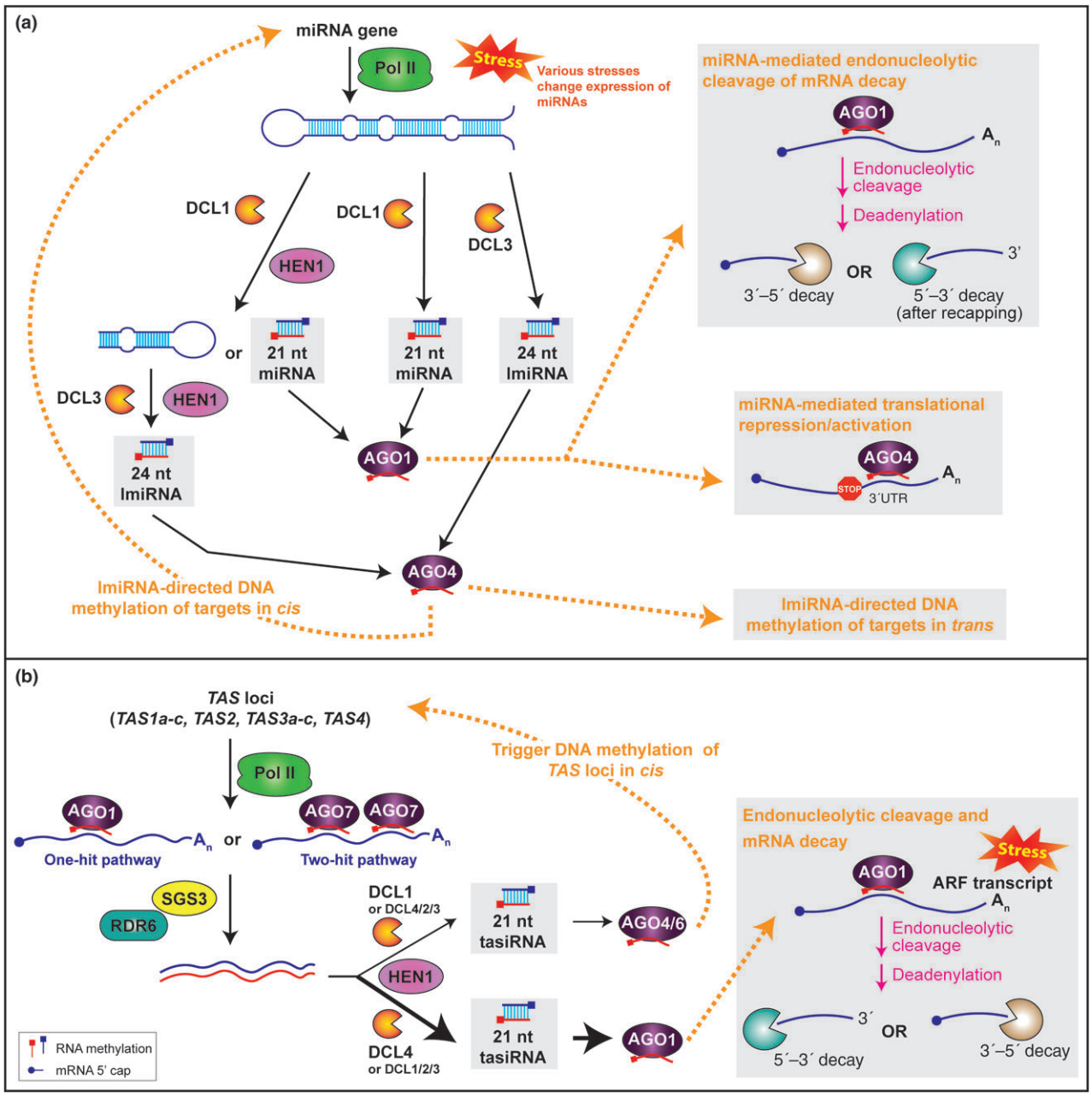

FIGURE 3 | Biogenesis of miRNAs, lmiRNAs, and tasiRNAs in *Arabidopsis*. (a) Schematics of miRNA and lmiRNA biogenesis. Most mature *Arabidopsis* miRNAs are 21 nt and produced by DCL1 from primary *miRNA* transcripts containing imperfect, self-complementary fold-back regions. However, DCL3 can process some *miRNA* hairpins, resulting in 24-nt lmiRNAs (left). Some *miRNA* transcripts can also give rise to both canonical miRNAs and lmiRNAs. The methyltransferase HEN1 also 2′-O-methylates the 3′ nucleotides of mature miRNA and lmiRNA duplexes to enhance their stability before they are loaded into AGO complexes. The miRNA duplex assembles with AGO1 into RISC, which then base pairs with mRNAs to induce miRNA-directed silencing through mRNA degradation or affects on translation. In contrast, lmiRNAs function with AGO4 and may strictly function in directing DNA methylation in *cis* and in *trans*, similar to the function of het-siRNAs. (b) Biogenesis and action of tasiRNAs in *Arabidopsis*. The productions of secondary phased 21-nt tasiRNAs are initiated by miRNA-mediated cleavage of *TAS* (*TAS1–4*) transcripts, which are transcribed by RNA Pol II. In the one-hit model, AGO1-loaded 22-nt miR173 and miR828 target a single site on *TAS1*, *TAS2*, and *TAS4* transcripts. In the two-hit model, AGO7-loaded 21-nt miR390 targets *TAS3* transcripts, which have two target sites. Cleaved transcripts are then converted into dsRNA by SGS3 and RDR6, and processed by DCLs before loading into AGO complexes. TasiRNAs can function in PTGS in *trans* and in TGS pathways in *cis*. Specific classes of 21-nt tasiRNAs are preferentially processed by DCL4 (with the redundant activities of DCL1/2/3) and loaded into AGO1 to direct target mRNA cleavage in *trans* post-transcriptionally. Other classes are processed predominantly by DCL1 (with DCL2/3/4 acting redundantly) and loaded into AGO4/6 complexes to recruit other RdDM effectors to mediate DNA methylation of TAS loci in *cis*.

auxin signaling.[80] MiR393 negatively regulates mRNAs encoding the F-box auxin receptors TIR1, AFB2, and AFB3, which normally promote expression of auxin-response genes and suppression of defense responses. The repression of auxin signaling by miR393 restricts growth of the bacterial pathogen *P. syringae*.

Phased, Secondary siRNAs: tasiRNAs and Other phasiRNAs

TasiRNAs belong to the group of secondary siRNAs generated in a phased pattern from noncoding tasiRNA-generating loci (*TAS* genes), which have thus far only been identified in plants. TasiRNAs control a much wider range of related mRNAs than individual miRNAs, including auxin response factor (*ARF*) transcripts and a large family of ARF and MYB transcription factors.[19] Their production is initiated by the miRNA-mediated cleavage of the transcripts from four *TAS* families in *Arabidopsis* (*TAS1–4*)[12–14] (Figure 3(b)). Two pathways generate tasiRNAs. In the one-hit pathway, AGO1 (guided by the 22-nt miR173 and miR828) cleaves single target sites present on the *TAS1*, *TAS2*, and *TAS4* transcripts.[12] After cleavage by AGO1 and RDR6-mediated dsRNA synthesis, tasiRNAs are processed by DCL4 from the miRNA-cleaved 3′ fragments. In the two-hit pathway, miR390 recognizes two sites in the RNA of *pri-TAS3*.[15] Processing of the dsRNA by DCL then begins from the end formed by miRNA-mediated cleavage. HEN1-methylated tasiRNAs are then loaded into AGO1 to regulate their mRNA targets in *trans* via PTGS.[5,81] TasiRNAs can also trigger DNA methylation of *TAS* loci in *cis*, suggesting an additional layer of regulation of tasiRNA expression.[16] The specific group of TAS-derived tasiRNAs that trigger DNA methylation of TAS loci in *cis* requires DCL1 and AGO4/6, rather than DCL4 and AGO1.

The activity of endogenous plant miRNAs is usually limited to one or a few cells; by contrast, tasiRNA regulation is non-cell-autonomous. The tasiRNA-initiating miRNAs are normally not mobile or only poorly mobile, but tasiRNAs display extensive cell-to-cell mobility. For example, tasiRNA-mediated regulation of *ARF* transcripts establishes a gradient of ARF expression throughout leaves and this gradient inversely correlates with the concentration of mobile tasiRNAs.[17,82,83]

Hormone signaling meditates stress responses and also acts in the regulation of plant development; therefore, plant responses to stress and developmental processes show substantial crosstalk. Analysis of mutants affecting tasiRNA synthesis under drought or high-salt conditions showed that the tasiRNA-ARF pathway acts to maintain proper flower morphogenesis even under stress conditions.[18] The extensive crosstalk among hormonal pathways suggests that changes in *ARF* expression modulated by tasiRNAs might broadly affect development and responses to stress. Although no tasiRNAs have been reported to act directly in acclimation to environmental stresses, some abiotic stresses do affect tasiRNA expression. For example, the abundance of a group of tasiRNAs from the *TAS1*, *TAS2*, and *TAS3* families increased significantly in plant roots under hypoxia.[84] Drought and salt stresses downregulate tasiRNAs and their precursor transcripts (*TAS1*, *TAS2*, and *TAS3*).[18]

In addition to tasiRNAs originating from *TAS* loci, a number of protein-coding loci can also give rise to miRNA-triggered secondary phasiRNAs in many plant genomes.[19] For example, the NB-LRR (nucleotide-binding, leucine-rich repeat) superfamily of plant disease resistance (R) genes, which encode immune receptors that recognize specific pathogen effectors and trigger resistance responses, both produce and are targeted by phasiRNAs.[85] The unregulated expression of NB-LRR genes in the absence of pathogen infection can trigger autoimmunity and inhibit plant growth; thus, the host downregulates the expression of NB-LRR genes through smRNAs. However, many bacterial and viral pathogens can suppress various host smRNA pathways and infections may cause reductions in both the accumulation and function of miRNAs triggering phasiRNAs production and phasiRNAs, thus leading to increases in the accumulation of NBS-LRR disease-resistance mRNAs and increases in immunity.[86] Thus, these phasiRNAs that target transcripts encoding NB-LRRs in *cis* and in *trans* serve as a feedback regulatory loop in the defense against pathogenic attacks.

Natural Antisense siRNAs

Nat-siRNAs are a class of siRNAs produced from natural antisense transcripts that are encoded locally in *cis* (*cis*-NATs) or encoded in *trans* (*trans*-NATs). *Cis*-NATs are a common feature of eukaryotic genomes, with 70% of annotated mRNAs in *Arabidopsis* associated with antisense transcripts, and similar percentages in mouse (72%) and human (~61–72%).[87–89] All plant nat-siRNAs examined to date function mainly at the post-transcriptional level. Many *cis*-NAT pairs in yeasts, plants, and animals show expression that is regulated by stresses or specific to developmental stages.[23,87,90,91] For example,

over 1300 NATs pairs are regulated either concordantly or discordantly by light in *Arabidopsis*. Moreover, genes for many light-regulated NATs occur at histone modification peaks, and changes in histone acetylation correlated with expression of NATs in response to light.[87] Various biotic and abiotic stresses also trigger production of nat-siRNAs from *cis*-NAT pairs in *Arabidopsis* and rice, and nat-siRNAs produced from these loci have been suggested to be important components of stress regulatory circuits.[20–22] The biogenesis pathways involved in nat-siRNA production are heterogeneous.[22,92] Biotic and abiotic stress-induced nat-siRNAs could be produced by DCL1 and/or DCL3. Surprisingly, the DCL3-dependent nat-siRNAs also showed a partial dependence on RDR2 and Pol IV; by contrast, most of the DCL1-dependent nat-siRNAs did not require RDRs and Pol IV. The partial RDR dependence indicates that these nat-siRNAs might primarily originate from dsRNA regions in overlapping sense–antisense transcripts. Amplification by RDRs may be a secondary step.[22]

An elegant example of the function of nat-siRNAs in stress comes from the nat-siRNAs derived from the *SRO5* and *P5CDH* *cis*-NAT pair, which participate in osmoprotection and management of oxidative stress in *Arabidopsis* exposed to salt stress.[21] *P5CDH*, a constitutively expressed gene, encodes an enzyme responsible for degradation of amino acid proline; *SRO5*, a salt stress-inducible gene, may function in counteracting reactive oxygen species (ROS). Induction of *SRO5* by salt stress creates dsRNA in the *SRO5-P5CDH* overlapping region and leads to the production the 24-nt siRNA by DCL2 (Figure 4(a)). The 24-nt siRNA guides the initial cleavage of the *P5CDH* transcript and sets the phase of the subsequently produced 21-nt nat-siRNAs formed by DCL1. Both 24-nt and 21-nt nat-siRNAs can further cleave *P5CDH* transcripts, thus downregulating *P5CDH* expression and leading to accumulation of proline, which contributes to the plant's ability to tolerate excess salt. *P5CDH* and *SRO5* appear to be functionally linked. Downregulation of *P5CDH* causes proline accumulation, which improves salt tolerance and also induces increased production of ROS in response to salt; SRO5 can counteract this increase in ROS. Thus, regulation of *P5CDH* and *SRO5* by nat-siRNAs functions in a regulatory loop controlling ROS and the response to salt stress.

Nat-siRNAs can also participate in antibacterial defenses in plants. For example, infection of *Arabidopsis* with the bacterial pathogen *P. syringae* specifically induces the 22-nt ATGB2 nat-siRNA derived from the overlapping region of NAT pair of *AtGB2* and *PPRL*.[22] Induction of the ATGB2 nat-siRNA leads to the silencing of the antisense gene *PPRL*, a putative negative regulator of RPS2-mediated disease resistance signaling, thus contributing to immunity.

Nat-siRNAs also occur in animal systems.[93–95] Many co-expressed *cis*-NAT gene pairs have also been identified in the mammalian brain, and a number of *cis*-NAT genes participate in synaptic signaling and in Alzheimer disease pathways.[91] Many nat-siRNAs are also upregulated in olfactory discrimination training, suggesting that they may participate in learning and memory.[94] The *cis*-NAT pair *LRRTM1* and *Ctnna2* functionally associates with susceptibility to schizophrenia and can generate nat-siRNAs.[94,96,97] Interestingly, *LRRTM1* is an epigenetically regulated paternally imprinted gene and hypomethylation at the *LRRTM1* promoter was shown to be a risk factor for the development of schizophrenia. The changes in DNA methylation patterns of *LRRTM1* have a unique role in brain development and in plasticity underlining learning, memory, and cognition. However, whether *LRRTM1-Ctnna2* nat-siRNAs affect epigenetic regulation of *LRRTM1* or whether any mammalian siRNAs (except piRNAs) participate in DNA methylation remain unknown. It also appears that all experimentally analyzed plant nat-siRNAs function mainly post-transcriptionally.

Long siRNAs

Pathogen infection in *Arabidopsis* also induces lsiRNAs, which are 30–40-nt long and derived from protein-coding genes and/or NAT pairs.[24] For example, AtlsiRNA-1 is specifically induced upon infection with *P. syringae* and derived, similar to nat-siRNAs, from the overlapping *cis*-NAT pair formed by the transcripts of the smRNA-generating gene *SRRLK* and the 3′ UTR of the antisense *AtRAP* gene (Figure 4(b)). AtlsiRNA-1 is primarily generated by DCL1, but it requires HYL1, HEN1, and HST1 for its biogenesis, and functions in complex with AGO7. DCL4, Pol IV, and RDR6 also act in the formation of AtlsiRNA-1, possibly in the secondary amplification step. Genetic analysis showed that AtlsiRNA-1 silences *AtRAP* upon infection.

Knockout mutants of *SSRLK*, the sense NAT transcript producing AtlsiRNA-1, showed no significant difference from wild type in disease resistance. However, *SSRLK*, encoding a receptor-like kinase, may function redundantly or may contribute indirectly to defense by inducing AtlsiRNA-1 from the antisense transcript. In contrast to *SSRLK*, *AtRAP*,

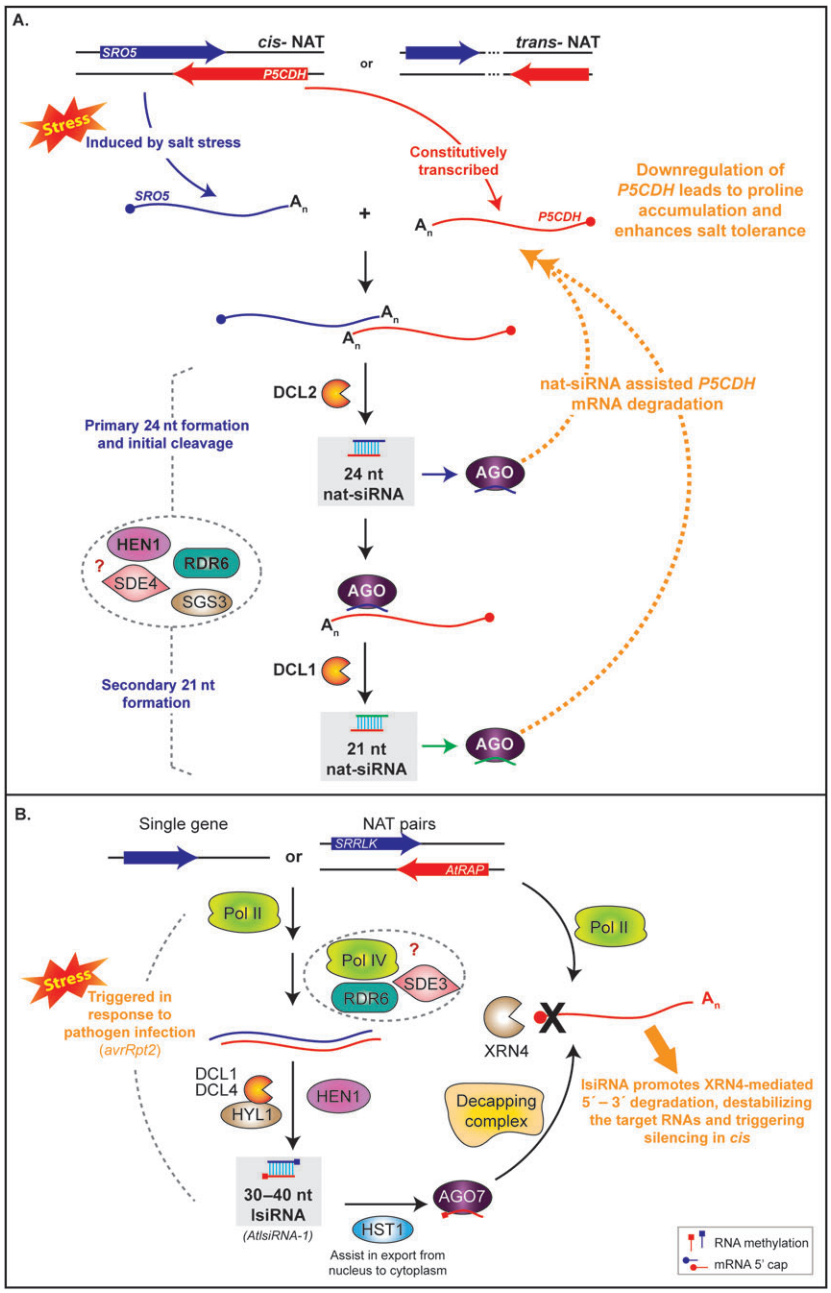

FIGURE 4 | Biogenesis and action of nat-siRNAs and long siRNAs in *Arabidopsis*. (a) Nat-siRNA processing from the *SRO5-P5CDH* gene pair and nat-siRNA function in a regulatory loop in response to salt stress. Nat-siRNAs can originate both from *cis*-NAT and *trans*-NAT transcript pairs. Salt stress leads to accumulation of reactive oxygen species (ROS), which can cause oxidation of DNA, proteins, and lipids, and induce the expression of *SRO5*. This triggers a series of nat-siRNA processing steps, resulting in the downregulation of *P5CDH*. The primary *SRO5* and *P5CDH* transcripts form dsRNA in their overlapping region, leading to production of 24-nt nat-siRNAs by DCL2. The initial cleavage of the *P5CDH* mRNA by 24-nt nat-siRNAs causes phased generation of 21-nt nat-siRNAs by a DCL1-dependent mechanism and additional cleavage of the *P5CDH* transcript. RDR6, SGS3, and Pol IV may contribute to the formation of both 24- and 21-nt SRO5-P5CDH nat-siRNAs. The 24-nt and 21-nt nat-siRNAs mediate the downregulation of *P5CSH* mRNAs, leading to proline accumulation, which contributes to salt tolerance. However, it also causes accumulation of the proline catabolic intermediate P5C and thus accumulation of ROS; ROS can harm cells, but also act as a signal that activates stress responses. SRO5 may counteract the accumulation of ROS, thus fine-tuning ROS levels and the resulting stress response. Adapted from Ref 21. (b) The biogenesis and mechanism of action of AtlsiRNA-1. LsiRNAs are 30–40 nt in length. AtlsiRNA-1 originates from the *SRRLK-AtRAP* NAT pair in response to infection by bacterial pathogens carrying the avrRpt2 effector and forms a complex with AGO7. AtlsiRNA-1 biogenesis requires DCL1, HYL1, HEN1, and HST1; DCL4, Pol IV, and RDR6 may also function in secondary amplification of AtlsiRNA-1. AtlsiRNAs may destabilize mRNAs via promoting decapping and subsequent XRN4-mediated 5′→3′ degradation in *cis*.

the antisense transcript producing AtlsiRNA-1, may be involved in regulating resistance responses. *AtRAP* expression is reduced in response to infection and its knockout mutants exhibit enhanced disease resistance. Genetic analysis showed that AtlsiRNA-1 silences *AtRAP* upon infection. AtlsiRNA-1 may destabilize the *AtRAP* transcript by promoting its decapping and 5′→3′ degradation by XRN4. Therefore, AtlsiRNA-1 may induce disease resistance by downregulating a factor that negatively regulates plant basal defense responses.

SmRNAs IN DNA DOUBLE-STRANDED BREAK REPAIR

Emerging evidence indicates that smRNAs play a direct role in repairing DNA damage that results from genotoxic stresses, distinct from the other functions of smRNAs in the nucleus. In this section, we examine the siRNAs produced near DNA DSBs and their similarities to other types of smRNAs.

DNA DSB-Induced siRNAs (diRNAs)

Many stresses, including UV light and ionizing radiation, cause DNA DSBs, which lead to genomic instability and, if unrepaired, to cell death. Eukaryotes have evolved complex and coordinated DNA damage response (DDR) and DSB repair pathways to combat DSBs; these pathways include nonhomologous end joining (NHEJ) and homologous recombination (HR), which were once thought to solely depend on protein factors.[98] However, accumulating reports suggest that smRNAs also participate in DSB repair. These smRNAs include DSB-induced diRNAs in *Arabidopsis* and human, QDE-2-interacting qiRNAs derived from rDNA repeats in *Neurospora crassa*, and possibly the repeat-associated rasiRNAs (analogs of mammalian piRNAs) in *Drosophila*.[25,99–102]

In *Arabidopsis*, 21-nt diRNAs are generated in the vicinity of DSB sites.[25] Their biogenesis requires Pol IV, Pol V RDR2/6, DCLs, and PI3 kinases, which primarily respond to stalled replication forks. AGO2 then uses diRNAs to mediate DSB repair through HR in *cis* (Figure 5). Studies in other systems showed that similar classes of DICER and DROSHA-dependent diRNAs (sometimes referred as DDRNAs in human cells) produced from sequences flanking DSB sites can directly rescue formation of DDR foci, further supporting the importance of smRNAs in DSB repair.[25,99,102]

Mutations in components of the diRNA biogenesis pathway significantly reduce DSB repair efficiency in *Arabidopsis* and human cells.[25] The components of diRNA biogenesis in *Arabidopsis* are known mainly as players in the RdDM pathway, in which siRNAs guide *de novo* cytosine methylation near the site of their biogenesis (described above in het-siRNAs section). However, DNA methylation levels at the DSB sites do not change and mutations in *AGO4* and *DRM2* (encoding a DNA methyltransferase) do not compromise the efficiency of DSB repair in *Arabidopsis*, indicating that while diRNA biogenesis requires RdDM components, the diRNAs do not function through the RdDM pathway to repair DSB.

One of the earliest events in response to DNA damage around DSB sites is the phosphorylation of histone variant H2AX to γ-H2AX, which facilitates recruitment and retention of DSB repair and chromatin remodeling factors.[103,104] However, depletion of diRNAs did not affect formation of γ-H2AX foci, indicating that diRNAs do not affect γ-H2AX accumulation. Moreover, these observations show that *Arabidopsis* diRNAs likely act downstream or parallel to γ-H2AX in the recruitment of DDR components for repair of DSBs.

RNA Pol V, which generates nascent scaffold transcripts in RdDM, is also required for DSB repair in *Arabidopsis*. Therefore, the diRNA–AGO2 complexes could function similarly to het-siRNAs to guide protein complexes (DDR components, repair complex, and possibly histone-modifying complexes) via base pairing to Pol V scaffold transcripts produced near DSB sites (Figure 5). Recent evidence found that human AGO2 guided by diRNAs physically interacts with and promotes recruitment and/or retention of Rad51, a DNA recombinase that binds to DNA at DSB sites and plays a major role in HR during DSB repair. The catalytic activity of AGO2 and diRNA binding have essential functions in recruitment of Rad51 and repair in HR,[33] further supporting the model in which AGO2–diRNAs complexes guide the DSB repair machinery.

Discovery of smRNAs involved in DSB repair showcases how diverse organisms from fungi to plants to humans can share a conserved mechanism that defends against DNA damage, one of the most lethal environmental stresses, despite the very different mechanisms of biogenesis of these DSBR-participating smRNAs.

REGULATION OF PRE-mRNA SPLICING BY NONCODING RNAs AND THE RNAi MACHINERY

Survival under stress conditions requires rapid reprogramming of gene expression and much of this

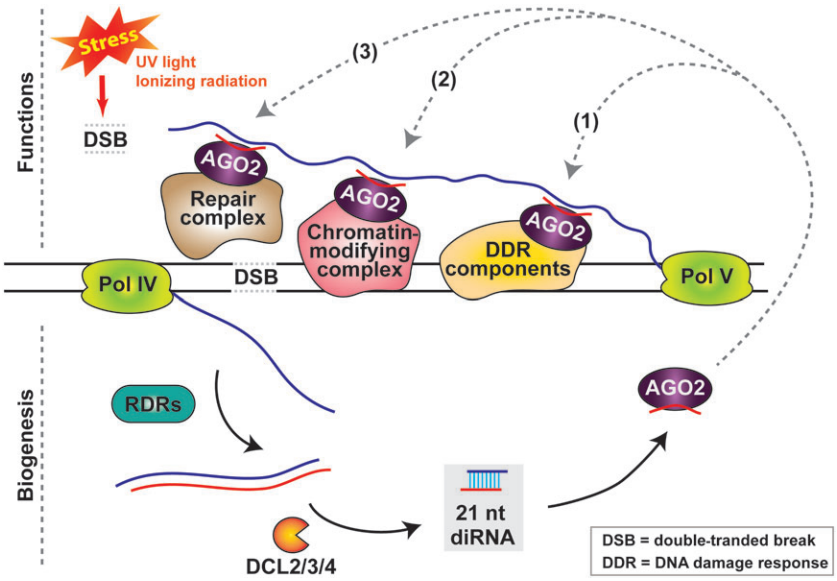

FIGURE 5 | A model for diRNA-mediated DSB repair in Arabidopsis. In the vicinity of DSB sites, ssRNAs are generated, presumably by RNA Pol IV. SsRNAs are then converted into dsRNAs by RDR or through bidirectional transcription. DCL2/DCL3/DCL4 processes the dsRNAs into mature diRNAs, which are subsequently incorporated into AGO2. AGO2–diRNA complexes localize to the DSB site through interaction with scaffold transcripts made by Pol V. The AGO2–diRNA complexes may activate the DNA damage response (DDR) by recruiting DDR components (1), and may modify local chromatin by recruiting chromatin-modifying components (2) or enable repair of the DSB by recruiting repair proteins (3). Adapted from Ref 25.

reprogramming occurs at the step of pre-mRNA splicing.[43,105,106] Even under normal conditions, almost all intron-containing genes in humans and over 60% of intron-containing genes in *Arabidopsis* undergo AS; environmental stresses trigger massive changes in the patterns of splicing in all organisms, including plants.[43,107–113] The crucial functions of splicing and smRNAs, two RNA-based pathways, in quick, responsive regulation of gene expression in eukaryotes indicate that these pathways may connect. However, examples of these pathways intersecting have remained limited, until recently. Now, emerging evidence indicates that noncoding RNAs (ncRNAs), including small and long ncRNAs (lncRNAs), and the RNAi machinery participate in the regulation of pre-mRNA splicing in various species. In this section, we describe our emerging understanding of the roles of smRNAs and RNAi in regulating splicing.

Mechanism of Splicing

Splicing is performed by the spliceosome, a ribonucleoprotein machine consists of five small nuclear RNAs (snRNAs) assembled into small nuclear ribonucleoproteins (snRNPs) and >100 non-snRNP proteins. Splicing proceeds via stepwise recognition of the short *cis* elements on pre-mRNAs by snRNPs through base-pairing interactions. Every intron has the minimal core splicing motifs, the 5′ splice site (5′SS), the 3′ splice site (3′SS), the branch point (BP) with its conserved A residue responsible for catalysis, and the polypyrimidine tract (PPT) located between the BP and the 3′SS[35,114] (Figure 6(a)). These core signals are recognized by the components of the spliceosome and additional auxiliary proteins participating in splicing, among which members of the serine/arginine-rich (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) protein families constitute the two main classes of splicing factors.[36] However, the minimal sequences are insufficient to define the correct splice sites and thus pre-mRNA molecules also carry a plethora of additional exonic/intronic *cis*-regulatory elements necessary for fidelity and efficiency of splicing; these elements constitute the 'splicing code.'[115] These *cis*-elements are recognized by *trans*-acting factors and together they act as regulators of constitutive splicing and AS (Figure 6(a)). Moreover, many additional splicing factors regulate AS in specific cell types, developmental stages, and in response to different stimuli.[112,115,116]

The Role of Chromatin Structure and Pol II Elongation Rate in the Regulation of Splicing

Although there are some organism-specific differences, splicing proceeds predominantly co-

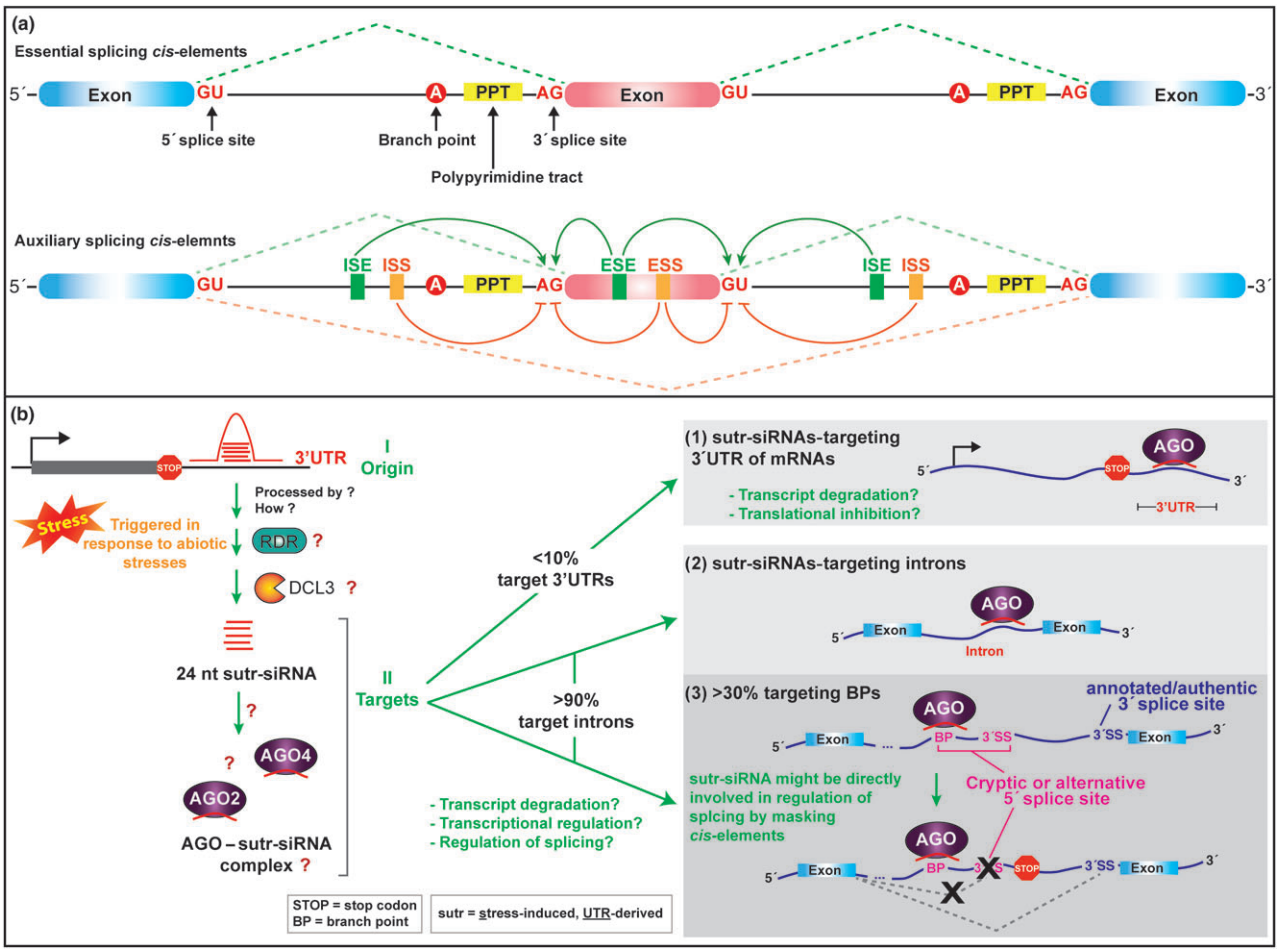

FIGURE 6 | Regulation of pre-mRNA splicing by smRNAs. (a) Schematics of splicing. Upper panel, the exon–intron architecture of eukaryotic genes. Exon sequences are boxed, constitutive exons are shown in blue, and alternative exons are in pink; black lines represent introns. The minimal essential core splicing *cis*-elements, 5′ splice site (5′SS) with invariant GU, 3′ splice site (3′SS) with invariant AG dinucleotides, and branch point sequence (BP) are indicated and present in every intron. The polypyrimidine tract (PPT) is a pyrimidine-rich sequence located between the BP and 3′SS. Spliceosome components are recruited to the pre-mRNA molecule and splicing proceeds via stepwise interaction of spliceosomal snRNPs recognizing the core splicing *cis*-elements on pre-mRNAs through base-pairing interactions. Lower panel, simplified schematics of the *cis*-acting 'splicing code.' Multiple additional exonic and intronic *cis*-regulatory elements, termed the 'splicing code,' define the correct splice sites and participate in regulation of alternative splicing. Intronic/exonic splicing enhancers, ISEs/ESEs, are marked in green and intronic/exonic splicing silencers, ISSs/ESSs, are marked in orange. Inclusion or skipping of alternative exon/s is regulated in a combinatorial manner by the relative strength of enhancers and silencers, which are bound by splicing *trans*-acting factors. Green arrows indicate enhancing, orange arrows indicate silencing processes. (b) Model for the mechanism of *Brachypodium* sutr-siRNAs action. 3′ UTRs of *Brachypodium* coding genes give rise (by unknown mechanisms) to mostly 24-nt sutr-siRNAs in response to various abiotic stresses (left panel). Sutr-siRNAs have potential different groups of *trans*-targets in the genome: 10% of sutr-siRNAs are predicted to target 3′ UTRs of other genes (panel 1, top right), which would be consistent with these sutr-siRNAs acting in translational regulation or mRNA stability of their targets. Over 90% of sutr-siRNAs target intronic regions (panels 2 and 3, middle and bottom right). The indicated annotated/authentic 3′SS is the splice site used to produce full-length protein. Over 30% of intron-targeting sutr-siRNAs target potential intron regulatory regions such as PPT and BP sequences. Sutr-siRNAs target PPTs and BPs of splice sites, which could be cryptic or alternative, marked as an additional 3′SS (panel 3, bottom right). The choice of these additional/alternative splice sites would lead to introduction of a premature stop codon downstream of that splice site, resulting in either a alternative short splice isoform or, most likely, producing RNA substrate for nonsense-mediated degradation, suggesting that sutr-siRNAs might be involved in regulation of splicing by blocking cryptic *cis*-elements from being recognized by U2 snRNP and other splicing proteins during stress conditions and thus promote the selection of the correct splice site.

transcriptionally[117,118] and is functionally linked to transcription and chromatin. The manner and order in which splicing factors are recruited to *cis* elements on the pre-mRNA during transcription also affect the choice of splice sites and determine the outcome of splicing, thus the rate of RNA Pol II elongation can

affect many, although not all, AS events.[119] Splicing is also linked to chromatin state and various studies demonstrated that chromatin structure can influence AS, which was proposed to occur via two nonmutually exclusive mechanisms: a kinetic coupling model and a chromatin-splicing adaptor model.[39] In the kinetic coupling model, chromatin structure, as influenced by DNA methylation and histone modifications, also affects the elongation rate of RNA Pol II. As a consequence, different Pol II elongation rates and Pol II pausing can affect the accessibility of various *cis*-regulatory elements on the pre-mRNA molecule and thus influence splicing patterns.[120–122] In the chromatin-splicing adaptor model, chromatin and chromatin-binding proteins that interact with specific epigenetic modifications can also recruit RNA-binding proteins, which are then transferred to the pre-mRNA, thereby influencing splicing decisions.[123–125] A number of recent studies have demonstrated that human siRNAs can regulate AS by mediating the formation of facultative heterochromatin and affecting the Pol II elongation rate, as described below.

DNA methylation is another key epigenetic modification used in mammalian and plant genomes to regulate transcription, where it is normally associated with transcriptional repression while in gene bodies it is usually associated with high expression levels.[31,126,127] In humans, honeybees, and *Arabidopsis*, DNA methylation is enriched in exonic regions relative to introns, suggesting it to be a marker for exon definition.[126,128,129] Emerging evidence indicates that DNA methylation is important for exon selection in splicing and for the regulation of AS.[130,131] Work in mammals also demonstrated that DNA methylation could inhibit or promote the recognition of alternatively spliced exons via three major DNA- and chromatin-binding factors: CTCF and MeCP2, which affect AS via modulating the Pol II elongation rate,[132,133] and HP1, which recruits splicing factors to bind to the methylated DNA and to the pre-RNA precursor.[131] Plant smRNAs are the main players in establishing *de novo* DNA methylation in plants. While it is very likely that the role of gene body methylation in regulating AS might be conserved between plants and animals, it is not known whether plant smRNAs play any role in differential exon–intron DNA methylation. We refer the reader to several recent, excellent reviews describing the intimate connection between chromatin structure and Pol II elongation in the regulation of splicing.[134–136]

Emerging Connections Between ncRNAs, the RNAi Machinery, and Regulation of Splicing in Plants

Recent work has implicated various ncRNAs in regulation of splicing. LncRNAs can serve as decoys by mimicking targets of regulatory proteins or RNAs involved in splicing, thus titrating them away and preventing or limiting their access to the targets. For example, the *Arabidopsis* lncRNA ASCO binds to regulators of AS (nuclear speckle RNA-binding proteins), changing the patterns of AS in the regulation of root development.[137,138] The role of the ASCO lncRNA as a hijacker of RNA-binding proteins places it in a similar functional category with a number of other lncRNAs, such as the human MALAT1, which regulates AS by controlling the activity of SR splicing proteins, via interfering with their phosphorylation,[139] and sno-lncRNAs, a group of intron-derived lncRNAs that associate with the Fox family of splicing regulators, which leads to altered splicing patterns of Fox-regulated pre-mRNAs.[140]

In plants, a direct link between smRNA pathways and pre-mRNA splicing has remained elusive. However, recent work has identified miRNA-binding sites in introns of plant genes, suggesting that these miRNAs could participate in cleavage of pre-mRNAs.[141] Also, in *Arabidopsis*, the expression of the miRNAs was reciprocally changed when their precursor transcripts were alternatively spliced in high temperatures and ABA treatments.[142,143]

More recent work in *Brachypodium distachyon*, a model system for biofuel grasses, identified a new group of stress-induced smRNAs with the potential to participate in the regulation of splicing.[28] These 24-nt long smRNAs, termed sutr-siRNAs (for stress-induced, UTR-derived siRNAs), arise from the 3′ UTRs of a group of genes in response to various abiotic stresses (Figure 6(b)). How the sutr-siRNAs are produced and which components of plant smRNA biogenesis pathway participate in their production remains unknown. *Arabidopsis* has 4 DCLs and 10 AGO proteins, each of which generally acts with a specific smRNA type.[144,145] DCL3 generates the majority of 24-nt siRNAs in *Arabidopsis*; therefore, the *Brachypodium* homolog of DCL3 might act in sutr-siRNA biogenesis. Also, most sutr-siRNAs have a 5′A, suggesting that if they act in RNAi pathway they preferentially load into the *Brachypodium* AGO2 and AGO4 homologs.

Intriguingly, over 90% of sutr-siRNAs can potentially target the intronic regions of many pre-mRNAs in *trans*. The bioinformatics analyses predicted that the intron-targeting sutr-siRNAs are

complementary to the sites characteristic of intronic PPTs, which serve as binding sites for U2AF65, an auxillary U2 snRNP splicing protein, early during spliceosome assembly.[35,146,147] Moreover, a third of sutr-siRNAs have sequence complementarity to predicted BPs, key intronic *cis*-element participating in the first transesterification step in the splicing reaction. Unexpectedly, analysis of potential targets of the sutr-siRNAs showed that these sutr-siRNAs did not target the BPs of the major annotated splice sites (those expected to be used to produce functional full-length proteins). Instead, the bioinformatics analysis predicted that the sutr-siRNAs targeted BPs upstream of the annotated major splice sites; these sites could potentially be alternative, decoy, or cryptic splice sites, also known as pseudo splice sites, the splice sites that match the consensus motifs but are rarely selected for splicing in the target introns (Figure 6(b)). The use of the splice sites targeted by sutr-siRNAs would introduce a stop codon downstream of the targeted splice sites and thus could produce either short AS variants or aberrant transcripts. Introduction of a premature stop codon could result in targeting of these AS or aberrant transcripts for nonsense-mediated decay and thus downregulate their expression.[148,149] These tantalizing results raise many intriguing questions about the mechanism of sutr-siRNA action. Addressing these questions will require substantial additional research using mutants that eliminate or suppress the expression of a specific sutr-siRNA, or by using experimental systems involving *in vitro* splicing extracts.

Connections Between RNAi and Splicing in Animals

Recent work in *Drosophila* and humans has reported examples of regulation of AS through smRNAs.[37,40,150,151] Human siRNAs can regulate AS by mediating the formation of facultative heterochromatin, increasing repressive histone modifications H3K9me2 and H3K27me3.[37,40] In these cases, mammalian AGO1 and AGO2 reduced the elongation rate of RNA Pol II and facilitated spliceosome recruitment, promoting inclusion of variant exons and thus affecting AS.[37,40] RNAi components and AGO proteins could also play a more general role in the nucleus. Genome-wide studies demonstrated that *Drosophila* AGO2 binds both promoter regions and G-rich motifs near AGO2-sensitive splice sites, and regulates AS as well as transcription of AGO2-associated genes,[41,152] while human AGO1 interacts with Pol II and binds to transcriptionally active promoters.[153]

More recent work found that human AGO1 also affects both alternative and constitutive splicing,[42] as AGO1 binds to enhancer RNAs (eRNAs), lncRNAs produced from transcriptional enhancers and implicated in transcriptional activation of genes regulated by these enhancers.[154] Interestingly, the interaction of AGO1 with eRNAs did not regulate transcription of the genes neighboring these enhancers; paradoxically, it regulated alternative and constitutive splicing events,[42] indicating that RNAi-mediated regulation of splicing could be even more complex than previously thought.

Potential Mechanism of Action of sutr-siRNAs

Although work in animal systems provides some insights, the mechanisms of action of sutr-siRNAs remain unclear. The approaches used in characterization of sutr-siRNAs cannot rule out the possibility that sutr-siRNAs could act in connecting splicing decisions with epigenetic modifications, as was shown for animal RNAi, or in degradation of pre-mRNAs. However, the fact that sutr-siRNAs target splice sites that could produce AS or aberrant transcripts subjected to nonsense-mediated decay (Figure 6(b)), which would downregulate expression of the involved gene, suggests that (Figure 6(b)) the sutr-siRNAs could act by masking *cis*-elements and preventing the splicing machinery from recognizing these BPs at the target splice site, thereby making sure that the splicing machinery uses the correct splice site.

One of the most intriguing genes predicted to be targeted by sutr-siRNAs is the *Brachypodium* ortholog of *Arabidopsis XRN4*, the functional homolog of yeast *XRN1*, encoding a $5'\rightarrow3'$ exoribonuclease that degrades uncapped mRNAs.[155,156] In *Arabidopsis*, XRN4 acts as silencing suppressor, a regulator of developmental and biotic stress response pathways,[156–159] and also plays an important role in the heat-sensing pathway.[160] AtXRN4 function is needed during heat shock as about 25% of the *Arabidopsis* transcriptome undergoes rapid XRN4-dependent degradation when the plants undergo global reprogramming of gene expression triggered by stress. In accord, *xrn4* mutant plants cannot acclimate to heat stress and the functional AtXRN4 is required for thermotolerance of plants during prolonged exposure to high temperatures.[160] Therefore, in the case of *Brachypodium XRN4*, sutr-siRNAs produced under stress conditions could act to ensure that plants use the correct splice site, producing functional XRN4 and helping the plant to survive.

Regulation of splicing largely involves the recruitment of the splicing machinery to specific locations or preventing access to these sites; it can also involve modulating interactions between splicing components, such as snRNPs. Although the mechanisms by which endogenous sutr-siRNAs affect splicing remain to be elucidated, many experimental and therapeutic approaches have used exogenous oligonucleotides that can bind to intronic *cis*-elements to alter pre-mRNA splicing pattern by blocking access of key splicing factors and thus redirecting splicing to a different splice site.[161–163] Studies by Kishore and Stamm[164,165] implicated the endogenous mammalian snoRNA HBII-52 and the smRNAs processed from it in splice-site selection via base-pairing, providing evidence that endogenous smRNAs can also modulate splicing.

Although sutr-siRNAs are produced in response to various abiotic stresses and over half of them exhibited >10-fold increases in levels in response to stress, these smRNAs are also likely produced under normal conditions, as one of the parameters used to identify sutr-siRNA-producing 3′ UTRs was that they produce sutr-siRNA in both stressed and unstressed plants.[28]

Why Are sutr-siRNAs Induced Specifically During Stresses?

Stresses trigger profound changes in the spectrum of proteins that need to be expressed by the cells in order to survive; implementation of these changes requires an arsenal of mechanisms acting to drastically alter and fine-tune gene expression. For example, during the response to stresses, eukaryotic cells from yeasts to humans normally repress general protein synthesis but upregulate the synthesis of chaperones and other specific stress response proteins required for organism survival.[166] During the global reprogramming of gene expression under stress, various mechanisms also regulate gene expression at the level of pre-mRNA splicing; for example, all eukaryotes implement profound changes in the patterns of AS during stresses, leading to production of different splice variants and thus proteins with different functions required for survival.[105,113,167]

Studies of splicing regulation during heat shock showed general inhibition of splicing in various systems. However, genes encoding proteins required for survival under stress, such as protein folding and oxidation reduction, continued to be spliced efficiently, indicating existence of mechanisms differentially regulating splicing under stress.[113] Interestingly, genome-wide studies conducted in a mammalian system indicated that the inhibition of splicing during heat shock mainly affected transcripts that were spliced post-transcriptionally, not transcripts that were spliced predominantly co-transcriptionally.[113] During stress, various stress-related proteins, hnRNPs, SR proteins, and other splicing factors are recruited to nuclear stress bodies (nSBs), the sites of active transcription directed by the major heat-shock transcription factor HSF1.[167] These specialized transcription sites and recruitment of splicing factors to nSBs might serve as one of the mechanisms to protect mRNAs transcribed in nSBs from splicing inhibition and enable their splicing, suggesting that the regulation of splicing during stress also has a spatial aspect.

Interestingly, previous single-molecule imaging studies demonstrated that the catalysis of constitutive splicing is predominantly co-transcriptional, while catalysis of AS often occurs post-transcriptionally.[168] Given the importance of AS for survival under stresses, this observation raises many interesting questions about how post-transcriptionally spliced and/or AS RNAs, transcribed outside of nSBs, are protected during stresses.

The analysis of sequence motif frequency in introns of unaffected genes relative to introns in all other genes showed that the abundance of G-runs in introns also correlated with the escape from intron retention, suggesting that the presence of particular *cis*-regulatory motifs in introns may incorporate instructions for responses to stress and facilitate their splicing under stress.[113] Together, these various layers of regulation of pre-mRNA splicing in stress conditions indicate the existence of many different mechanisms acting to achieve regulation of pre-mRNA splicing under stress.

The changes in splicing during the stress response give intriguing clues to the potential functions of sutr-siRNAs, inviting further speculation as to whether they provide another layer of regulation in pre-mRNA splicing when organisms encounter stresses. During stress, chaperones segregate many protein factors to protect them from misfolding or degradation during stresses, making them unavailable.[169,170] In the stress response, endogenous smRNAs that bind to *cis*-regulatory elements or cryptic splice sites may prevent the splicing machinery from using these other splice sites, thereby improving the fidelity of splicing; this function may be particularly important under stresses. It is also possible that during stress, reliance on base-pairing interactions through smRNAs, rather than endogenous protein factors, becomes more important. However, determining whether sutr-siRNAs function in regulating AS, or ensuring the fidelity of splicing (via masking

cis-elements), or affecting DNA and/or chromatin modifications, or in distinguishing which transcripts should be spliced under stress (by degrading them), or in interpreting the vastly complicated splicing code, remains an exciting topic for future studies.

One intriguing question raised by the discovery of these smRNAs in *Brachypodium* is whether sutr-siRNAs are present in animals or other plants? It will also be interesting to determine if a similar group of endogenous smRNAs exists in different plant species or other organisms, but further experimental work will be needed to establish the mechanistic details of sutr-siRNAs biogenesis and actions. The sutr-siRNAs may provide a connection between RNAi and splicing, which may be triggered specifically in response to environmental stresses or when organisms face a strong threat. Thus, although the exact mechanisms of sutr-siRNA function remain to be elucidated, their mechanisms of action likely provide protection for cells under stress.

CONCLUSION

Plants have an incredibly rich variety of smRNAs, which do an outstanding job regulating gene expression and also participate in protecting plants from dangerous external conditions. These diverse mechanisms of action of plant smRNAs provide plants with powerful tools to induce and suppress expression of key stress-response genes by epigenetically regulating transcription, mRNA stability, and translation during the global reprogramming of gene expression required to survive stresses. Remarkably, recent work in plants, animals, and fungi continues to discover novel mechanisms of smRNA action beyond their classic functions in PTGS and epigenetic regulation; these mechanisms include the ability of smRNAs to mediate DSB repairs by guiding the repair machinery to specific sequences near DSB sites, the role smRNAs play in modulating AS in animals, and perhaps the functions of sutr-siRNAs, which might mask intronic *cis*-elements under stresses in plants.

The exciting connection shown in animal system between smRNAs and splicing pathways opens new immensely interesting areas of research, such as exploring the role DNA methylation plays in the regulation of AS in mammals. The intersection of RNAi and splicing pathways likely provides particularly valuable regulatory responses for organisms in situations that require speedy global reprogramming of gene expression. However, much still remains to be understood about the involvement of smRNAs and RNAi in the regulation of splicing in all systems. It would also be very interesting to find out if smRNAs and/or RNAi components are involved in the regulation of splicing in plants in a similar way to that observed in animals. Both plants and mammals extensively use DNA methylation in organization of their chromatin and regulation of gene expression (albeit with some organism-specific differences). While no smRNAs, except piRNAs, have been shown so far to regulate DNA methylation in animals, plant smRNAs are the main players in establishing *de novo* DNA methylation in plants. Therefore, an investigation of the connection between smRNAs, DNA methylation, and regulation of splicing warrants more attention, and how these mechanisms join forces when guarding against stresses, particularly in light of the numerous studies reporting that various environmental challenges commonly change the status of DNA methylation in plants. It will also be extremely interesting to uncover how sutr-siRNAs function in splicing and stress responses in plants and/or other organisms, to find out whether they act by affecting chromatin modifications, via masking regulatory intronic *cis*-elements, or by any other mechanisms while acting to guide protein factors via RNA complementarity to specific intronic *cis*-elements.

Future research will undoubtedly uncover new mechanisms by which smRNAs regulate stress responses in plants, and reveal the mechanistic and regulatory connections among the various pathways described above. Moreover, integrating the significant advances made in understanding the mechanism of pre-mRNA splicing (particularly in animal systems) and various mechanisms of smRNA action in plants, fungi, and animals will improve our understanding of how organisms in all kingdoms of life defend against stressful environments.

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