The Functions of RNA-Dependent RNA Polymerases in Arabidopsis

Matthew R. Willmann\textsuperscript{a,b}, Matthew W. Endres\textsuperscript{a,b}, Rebecca T. Cook\textsuperscript{a}, and Brian D. Gregory\textsuperscript{a,b,c,1}

\textsuperscript{a}Department of Biology, \textsuperscript{b}PENN Genome Frontiers Institute, \textsuperscript{c}Genomics and Computational Biology Graduate Program University of Pennsylvania, Philadelphia, PA 19104, USA

1Address correspondence to bdgregor@sas.upenn.edu

One recently identified mechanism that regulates mRNA abundance is RNA silencing, and pioneering work in \textit{Arabidopsis thaliana} and other genetic model organisms helped define this process. RNA silencing pathways are triggered by either self-complementary fold-back structures or the production of double-stranded RNA (dsRNA) that gives rise to small RNAs (smRNAs) known as microRNAs (miRNAs) or small-interfering RNAs (siRNAs). These smRNAs direct sequence-specific regulation of various gene transcripts, repetitive sequences, viruses, and mobile elements via RNA cleavage, translational inhibition, or transcriptional silencing through DNA methylation and heterochromatin formation. Early genetic screens in Arabidopsis were instrumental in uncovering numerous proteins required for these important regulatory pathways. Among the factors identified by these studies were RNA-dependent RNA polymerases (RDRs), which are proteins that synthesize siRNA-producing dsRNA molecules using a single-stranded RNA (ssRNA) molecule as a template. Recently, a growing body of evidence has implicated RDR-dependent RNA silencing in many different aspects of plant biology ranging from reproductive development to pathogen resistance. Here, we focus on the specific functions of the six Arabidopsis RDRs in RNA silencing, their ssRNA substrates and resulting RDR-dependent smRNAs, and the numerous biological functions of these proteins in plant development and stress responses.

INTRODUCTION

RNA silencing mechanisms control gene expression transcriptionally or post-transcriptionally in a sequence-specific manner (Baulcombe, 2004). In RNA silencing, self-complementary RNA fold-back structures or the production of intermolecular double-stranded RNA (dsRNA) gives rise to 20 – 24 nucleotide (nt) small RNAs (smRNAs) through the activity of DICER or DICER-LIKE (DCL) RNase III-type ribonucleases (Meister and Tuschl, 2004; Jones-Rhoades et al., 2006). These smRNAs comprise the sequence-specific effectors of RNA silencing pathways that negatively regulate genes, repetitive sequences, viruses, and mobile elements (Almeida and Allshire, 2005; Tomari and Zamore, 2005). The two major types of endogenous smRNAs in plants are microRNAs (miRNAs) and small interfering RNAs (siRNAs), the latter having several subclasses, including heterochromatic siRNAs (hsiRNAs), trans-acting siRNAs (ta-siRNAs), and natural antisense transcript-derived siRNAs (nat-siRNAs). These different types of smRNAs are differentiated from one another by the classes of genomic loci from which they arise, their distinct biosynthetic pathways, and their silencing targets (Baulcombe, 2004). smRNAs become incorporated into ARGONAUTE (AGO)-containing RNA-induced silencing complexes (RISC), which execute effective silencing of RNA species containing sequence complementarity (Voinnet, 2008).

In plants, siRNAs are typically derived from long dsRNA molecules synthesized by an RNA-dependent RNA polymerase (RDR) (Bartel, 2004; Baulcombe, 2004; Carthew and Sontheimer, 2009). In fact, RDRs were among the first RNA silencing components to be identified (Dalmay et al., 2000; Mourrain et al., 2000; Voinnet, 2008). These enzymes convert single-stranded RNAs into dsRNAs. RDRs can synthesize dsRNA by primer independent (Tang et al., 2003) or primer dependent means using an smRNA as a primer (Moissiard et al., 2007; Voinnet, 2008). In fact, smRNA primed RDR amplification is actually required for the formation of some secondary siRNAs (reviewed in Voinnet, 2008). The dsRNA products of RDRs are sliced by DICER into 20 – 24 base pair (bp) siRNA duplexes. Because the siRNAs that are in antisense orientation can direct the negative regulation of target transcripts from the same gene or other complementary sequences, the silencing is amplified. Thus, the involvement of RDRs in RNA silencing explains how robust, persistent negative regulation can be initiated by small inputs of RNA, including those from transposons or viruses.

Classification of RDRs

RDRs are defined by the presence of a conserved RNA-dependent RNA polymerase catalytic domain. These proteins are found in RNA viruses, plants, fungi, protists, and some lower animals, including \textit{Caenorhabditis elegans}, but are absent in \textit{Drosophila}, mice, and humans. There are three major clades of
RDRs — RDRα, RDRβ, and RDRγ — and, based on phylogenetic analysis, it is thought that one member of each was present in the most recent common ancestor of plants, animals, and fungi (Zong et al., 2009). While RDRα genes have been sequenced from all three kingdoms, RDRβ has only been found in animals and fungi and RDRγ only in plants and fungi. Thus, RDRβ was likely lost in the plant lineage, while RDRγ was lost in animals (Zong et al., 2009).

The model plant Arabidopsis thaliana possesses six identifiable RDRs (Wassenegger and Krczal, 2006). The RDRα genes have duplicated in plants to yield separate RDR1, RDR2, and RDR6 subgroups, with apparent functional diversification, and Arabidopsis has one of each type (Zong et al., 2009). Arabidopsis RDR1, RDR2, and RDR6 all share the C-terminal canonical catalytic DLDGD motif of eukaryotic RDRs and have orthologs in many plant species (Wassenegger and Krczal, 2006). Initially, these enzymes were studied because of their role in plant antiviral and transgene silencing, but it is becoming increasingly apparent that they have additional molecular functions, including the control of chromatin structure (RDR2) and the regulation of cellular gene expression (RDR6) (Figure 1). Arabidopsis also has three RDRγ genes (RDR3, RDR4, and RDR5; also called RDR3α–RDR3γ), which share an atypical DFDGD amino acid motif in the catalytic domain. To date, these three proteins have not been assigned functions, but the presence of at least one RDRγ gene in several other sequenced plant genomes, including rice (Oryza sativa), poplar (Populus trichocarpa), moss (Physcomitrella), and a lycophyte (Selaginella), as well as many fungi, suggests that one or more RDRγ proteins in Arabidopsis may have functional significance (Figure 1) (Wassenegger and Krczal, 2006; Zong et al., 2009). In this chapter, however, we will focus most of our attention on the functions of the three best-characterized Arabidopsis RDRs (RDR1, 2, and 6).

**RDRs in smRNA biogenesis**

RDR1, RDR2, and RDR6 are all known to function in the formation of dsRNA molecules that are subsequently processed into different types of siRNAs targeting specific endogenous loci (Voinnet, 2008). RDR2 is involved in the biogenesis of the most abundant endogenous siRNAs in plants — hsiRNAs. hsiRNAs are mostly 24 nt in length and are associated with heterochromatic and repetitive regions of the Arabidopsis genome, including pericentromeric regions and telomeres (Figure 2). These smRNAs direct

![Figure 1](https://bioone.org/journals/The-Arabidopsis-Book) The functions of Arabidopsis RDRs.

The model plant Arabidopsis possesses six RDRs that can be easily identified. RDR3, 4, and 5 share an atypical DFDGD amino acid motif in the catalytic domain. These three proteins have not been assigned functions to date. The better-studied Arabidopsis RDR proteins are RDR1, 2, and 6, whom all share the C-terminal canonical catalytic DLDGD motif of eukaryotic RDRs and have direct orthologs in many plant species.
sequence-specific cytosine methylation and histone modification (RNA–directed DNA methylation (RdDM)). The result of RdDM is heterochromatinization of targeted genomic DNA regions, thereby producing stable transcriptional gene silencing at the target loci (Chan et al., 2005; Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005; Qi et al., 2006; Zheng et al., 2007). RdDM is associated with the posttranscriptional silencing of endogenous transposons, retroelements, and other classes of repeats that are highly enriched within pericentromeric and other heterochromatic regions of the Arabidopsis genome (Chan et al., 2005; Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005; Qi et al., 2006; Zheng et al., 2007). RdDM is also responsible for siRNA-mediated DNA methylation and histone modifications at Arabidopsis telomeres, where it is required for the maintenance of telomeric heterochromatin (Vrbsky et al., 2010). This heterochromatization of Arabidopsis telomeres is not surprising given that it is a common feature of chromosome termini from many organisms (e.g. yeast, flies, and mammals) (Vrbsky et al., 2010).

Production of hsiRNAs likely begins when the plant specific DNA-dependent RNA polymerase IV (commonly referred to as Pol IV), with the assistance of CLASSY1, transcribes heterochromatic regions to produce single-stranded RNAs (ssRNAs). RDR2 then uses these transcripts as a template for dsRNA production. The dsRNAs are then processed by DCL3 into 24 nt hsiRNAs. The hsiRNAs are then methylated on their 3' ends by HEN1. The green circles on the ends of hsiRNAs denote methylation. The methylated hsiRNAs are bound by AGO4/6/9-RISC with target and silence particular genomic loci. Pol V likely generates non-coding transcripts from siRNA-targeting loci or intergenic regions just outside of the loci with the assistance of other known RdDM components, including DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3). These Pol V-dependent RNAs likely serve as a scaffold to recruit AGO4/6/9-RISC with a bound hsiRNA. Finally, DNA methyltransferases, including DOMAINS REARRANGED METHYLASE1 and 2 (DRM1 and DRM2) are recruited to the RdDM target loci by AGO4/6/9-RISC with a bound hsiRNA. Methylation by DRM1/2 is denoted by the orange circles with the M in the middle attached to the DNA molecule (grey helix) that is wrapped around nucleosomes (blue boxes).

Figure 2. The RNA-directed DNA methylation pathway of Arabidopsis.

A cartoon representation of the current model for the Arabidopsis RdDM pathway is shown. Briefly, Pol IV is believed to transcribe heterochromatic regions and produce a single-stranded transcript with the assistance of CLASSY1. Next, RDR2 uses these transcripts as a template for dsRNA production. The dsRNAs are then processed by DCL3 into 24 nt hsiRNAs. The hsiRNAs are then methylated on their 3' ends by HEN1. The green circles on the ends of hsiRNAs denote methylation. The methylated hsiRNAs are bound by AGO4/6/9-RISC with target and silence particular genomic loci. Pol V likely generates non-coding transcripts from siRNA-targeting loci or intergenic regions just outside of the loci with the assistance of other known RdDM components, including DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3). These Pol V-dependent RNAs likely serve as a scaffold to recruit AGO4/6/9-RISC with a bound hsiRNA. Finally, DNA methyltransferases, including DOMAINS REARRANGED METHYLASE1 and 2 (DRM1 and DRM2) are recruited to the RdDM target loci by AGO4/6/9-RISC with a bound hsiRNA. Methylation by DRM1/2 is denoted by the orange circles with the M in the middle attached to the DNA molecule (grey helix) that is wrapped around nucleosomes (blue boxes).
with the assistance of other known RdDM components, including DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) (Kanno et al., 2004; Kanno et al., 2008; Wierzbicki et al., 2008). In fact, these Pol V-dependent RNAs likely serve as sequence-specific scaffolds to recruit complementary hsiRNAs bound to AG04/6/9-RISC to the genomic locus (Wierzbicki et al., 2009; Law and Jacobsen, 2010). Finally, DNA methyltransferases, including DOMAINS REARRANGED METHYLASE1 and 2 (DRM1 and DRM2) are recruited to the RdDM target loci by the hsiRNA-bound AG04/6/9-RISC by a yet unknown mechanism (Figure 2) (Law and Jacobsen, 2010). In summary, the involvement of RDR2 in the RdDM pathway means that an RDR protein is necessary for maintaining genome integrity and heterochromatic silencing in both somatic and reproductive tissues of Arabidopsis.

Interestingly, Arabidopsis RDR2 has also been found to function in the generation of long siRNAs (23 – 27 nt) from the same sites of stem loop precursors that normally produce mature miRNAs (20 – 22 nt) (Chellappan et al., 2010). Like hsiRNAs, these miRNA-derived long siRNAs (IsiRNAs) are dependent on DCL3 and Pol IV for their biogenesis, and can associate with AG04 to direct DNA methylation to specific target loci. The regulatory targets of these siRNAs are often also targeted by the mature miRNA for transcript cleavage/degradation. In fact, the combination of IsiRNA-directed DNA methylation and miRNA-mediated target RNA cleavage/degradation is required to produce complete posttranscriptional silencing of specific regulatory targets (Chellappan et al., 2010). A similar class of smRNAs known as long miRNAs (lmiRNAs) is also processed from canonical miRNA (cmiRNA)-producing sites of stem loop precursors in rice (Wu et al., 2010). Although this class of miRNAs requires DCL3 for processing and can also direct AG04-dependent methylation to target loci, they are not dependent on rice RDR2 for their biogenesis (Wu et al., 2010). Therefore, it seems that different plants use distinct biogenesis mechanisms to produce these long smRNAs with similar regulatory functions. In summary, Arabidopsis RDR2 is required for the production of the most abundant class of siRNAs (hsiRNAs), as well as a surprising class of long siRNAs that originate from miRNA-producing stem loops.

RDR6 has the broadest range of defined functions and substrates, and is involved in the biogenesis of at least two additional types of siRNAs: ta-siRNAs and nat-siRNAs. ta-siRNAs are generated from non-protein-coding TRANS-ACTING siRNA (TAS) precursors that are targets of miRNAs (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005; Yoshikawa et al., 2005). In general, following miRNA-directed TAS cleavage by AG01-RISC, RDR6 (with the help of its interacting partner SUPPRESSOR OF GENE SILENCING3 (SGS3)) converts the cleavage products to dsRNA. The dsRNA formed from the 3′ cleavage product is successively processed by DCL4 in 21 nt increments from the initially cleaved end, thus resulting in phased siRNAs (Allen et al., 2005; Gasciollì et al., 2005; Yoshikawa et al., 2005; Adenot et al., 2006; Axtell et al., 2006; Fahlgren et al., 2006; Garcia et al., 2006). Once incorporated into an ARGONAUTE (AGO)-containing RISC, ta-siRNAs post-transcriptionally down-regulate protein-coding transcripts from unrelated loci by target cleavage, reminiscent of the miRNA-directed RNA silencing pathway (Figure 3) (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005; Yoshikawa et al., 2005).

TAS transcripts are different from typical miRNA targets in one of two ways that make them substrates for RDR6. For TAS3 there are two miR390 target sites, one that is cleavable by miR390-bound AG07-RISC (3′ end of transcript) and another that is not (5′ end of RNA) (Figure 3A). All current evidence suggests that the dual miRNA390 target sites, as well as the involvement of AG07-RISC, results in the recognition of TAS3 by RDR6, SGS3, and/or some other ta-siRNA biogenesis component, initiating ta-siRNA production (Axtell et al., 2006; Montgomery et al., 2008a). The ta-siRNAs produced from TAS3, once incorporated into an AG01-RISC, go on to target ARF3 and 4 transcripts for posttranscriptional silencing (Adenot et al., 2006; Fahlgren et al., 2006; Garcia et al., 2006). This method of secondary siRNA generation is referred to as the two-hit trigger mechanism (Axtell et al., 2006). ta-siRNA biogenesis from the other three TAS transcripts (TAS1, 2, and 4) begins with cleavage at a single site by AG01-RISC that is bound to a 22 nt miRNA (miR173 for TAS1 and TAS2 and miR828 for TAS4) (Montgomery et al., 2008b; Chen et al., 2010; Cuperus et al., 2010). These 22 nt miRNAs are one nucleotide longer than average Arabidopsis miRNAs, which are 21 nt. This size difference is thought to allow recognition of AG01-22 nt miRISC by RDR6, SGS3, and/or some other component of the ta-siRNA biogenesis machinery that can then initiate production of phased secondary siRNAs from these loci (Figure 3B) (Chen et al., 2010; Cuperus et al., 2010). Together, these results suggest that RDR6-dependent ta-siRNA biogenesis can be initiated by more than one mechanism.

RDR6 is also required for the biogenesis of nat-siRNAs (Borsani et al., 2005). Nat-siRNAs are processed from the overlapping sections of transcripts that are expressed from opposing promoters on opposite coding strands. One of the two transcripts is a non-coding RNA, while the other is a protein-coding mRNA. The overlapping portion of the transcript pair yields a double-stranded region. These dsRNAs are processed by the small RNA biogenesis components Pol IV and/or Pol V, RDR6, SGS3, and DCL1 to produce nat-siRNAs. There does appear to be some variability in the biosynthetic pathways of different nat-siRNAs, however. This difference may be related to the sizes of the active nat-siRNAs in each case, as only DCL1 is required in the instance where the biologically active nat-siRNA is 22 nt (Katyar-Agarwal et al., 2006), but both DCL2 and DCL1 are involved when 24 nt and 21 nt nat-siRNAs are produced (Borsani et al., 2005). In the latter example, the overlapping transcripts are first processed by DCL2 into 24 nt nat-siRNAs, RDR6 and SGS3 then make dsRNAs from the partially processed transcripts, and finally the dsRNAs are cut by DCL1 into 21 nt nat-siRNAs (Borsani et al., 2005).

In two of the three known examples of nat-siRNAs in Arabidopsis, expression of the non-coding RNA is induced by some biotic or abiotic stimulus. These nat-siRNAs act to repress the protein-coding transcript of the overlapping gene pair specifically in response to the stress (Borsani et al., 2005; Katyar-Agarwal et al., 2006). In the third example, the non-coding transcript is developmentally regulated, resulting in silencing of the overlapping protein-coding transcript only in specific cells (Ron et al., 2010). We will come back to each of these examples below during the discussion of the roles of RDRs in pathogen defense, abiotic stress responses, and development.

Recently, RDR6 was demonstrated to produce phased and unphased siRNAs from RNAs that do not appear to be targeted...
For initial cleavage by either mi-/siRNAs or overlapping transcription (Figure 3C) (Zheng et al., 2010). These findings suggest that other mechanisms exist in Arabidopsis by which RDR6 selects substrates for production of both phased and unphased siRNAs. In total, RDR6 can target >200 loci in Arabidopsis for production of phased or unphased siRNAs (Zheng et al., 2010). But much remains unknown about substrate selection by RDR6, the biosynthesis mechanism of these different types of siRNAs, and their functionality, if any, in plants.

Like RDR2 and RDR6, RDR1 also has effects on the endogenous populations of Arabidopsis siRNAs, but these have not been studied in as much detail (Kasschau et al., 2007). The major defined role of RDR1 is in the production and amplification of exogenous, virus-derived siRNAs (vsiRNAs) in infected plants (Donaire et al., 2008; Wang et al., 2010b). Additionally, a recent study suggests that RDR1 may protect plants against herbivore attacks (Pandey and Baldwin, 2007).

Here, our focus will be on the many processes that require RDR function in Arabidopsis, including various facets of plant development, as well as responses to biotic and abiotic stresses. We illustrate that RDRs, through their functions in smRNA biogenesis, are required for numerous aspects of plant biology.

**RDR PROTEINS IN ARABIDOPSIS DEVELOPMENT**

RDRs have been associated with several aspects of Arabidopsis development. Most of these roles are attributed to RDR6, the first and most-well-studied RDR to date in Arabidopsis (Elmayan et al., 1998). Mutations in RDR1 are reported to neither...
have morphological effects nor enhance those of rdr6 (Xu et al., 2006; Wang et al., 2010b), and mutants of RDR3, RDR4, and RDR5 have not been studied in detail. The only characterized developmental role for RDR2 in Arabidopsis is in the development of the female gametophyte (Olmedo-Monfil et al., 2010). By contrast, loss-of-function mutations of the RDR2 ortholog in maize (mediator of paramutation1, mop1) are reproducibly late flowering (Dorweiler et al., 2000) and have an abnormally shaped vegetative meristem (Jia et al., 2009). Several other phenotypes of mop1 — diminished stature, spindly stalks, feminized tassels, and reduced fertility — are stochastic, but are seen in two independent mutants, suggesting that the pleiotropy of mop1 is due to the mutation itself and not to the mobilization of transposons (Dorweiler et al., 2000).

The six RDRs in Arabidopsis have different developmental and stress-responsive expression patterns based on the At-TAX tiling microarray experiments (Figure 4) (Laubinger et al., 2008). (AtGenExpress data is not discussed in detail here because RDR3 and RDR4 are not represented on the ATH1 Affymetrix microarray.) For instance, RDR1 has expression peaks in old leaves and the inflorescence apex. RDR3 and RDR4 are most highly expressed in the inflorescence apex. RDR2, RDR6, and to a lesser extent RDR5 are pretty consistently expressed in the At-TAX development data (Figure 4A), though RDR5 is strongly enriched in dry seeds in the AtGenExpress experiments. Additionally, the RDR6 gene is the most responsive to various stresses (Figure 4B). In fact, this gene is induced in response to high temperatures, while it is repressed during prolonged exposure to salt or cold stress. Interestingly, the expression of RDR1 and RDR5 also decreases during long exposures to high salinity and cold temperatures. The expression of RDR2, RDR3, and RDR4 genes is not significantly affected by the various stresses tested in the At-TAX study (Figure 4B).

In the next few sections, we will consider the known roles of RDRs in a number of Arabidopsis developmental processes, most of which have been attributed to the role of RDR6 in the biogenesis of ta-siRNAs from TAS3 or TAS4. However, it is worth noting that the developmental variations in RNA expression patterns (Figure 4) and the phenotypes of mop1 in maize suggest that undiscovered roles for RDRs in Arabidopsis development may exist.

**RDR6 function in Arabidopsis vegetative phase change**

While RDR6 has been known to be important for post-transcriptional gene silencing since 1998 (Elmayan et al., 1998), developmental phenotypes were first assigned years later when it was identified as a heterochronic mutant, one that affects developmental timing (Peragine et al., 2004). The life cycle of an angiosperm shoot consists of embryonic, juvenile vegetative, adult vegetative, and reproductive developmental phases, each associated with the production of different lateral organs — cotyledons, juvenile leaves, adult leaves, and reproductive leaves and flowers, respectively. rdr6 was identified in a screen for mutants

![Figure 4](https://jsp.weigelworld.org/tileviz/)

**Figure 4.** The expression patterns of all six Arabidopsis RDR genes in numerous tissues and stress conditions from tiling microarray experiments. (A) Absolute expression values (average tiling microarray signal intensity) for all six Arabidopsis RDR genes in the indicated plant tissues. Veg. is short for vegetative. Inf. is short for inflorescence. (B) Absolute expression values (average tiling microarray signal intensity) of the six Arabidopsis RDR genes under the indicated abiotic stress treatment. The salt condition was growth in 200 mM NaCl for the indicated times in hours (hrs). The osmotic stress condition was treatment of Arabidopsis plants with 300 mM mannitol for the indicated length of time. ABA treatment was done at 100 μM for the indicated times. Cold and heat refer to exposure of Arabidopsis plants to 8°C and 30°C, respectively, for the indicated times. All data presented in this figure was taken from tiling microarray analysis of Arabidopsis plants that can be found at Tileviz (http://jsp.weigelworld.org/tileviz/) (Laubinger et al., 2008).
that transition from the juvenile vegetative to adult vegetative phase (vegetative phase change) earlier than wild-type plants (Peragine et al., 2004). In Arabidopsis, juvenile and adult leaves can be distinguished by the absence or presence of abaxial trichomes; juvenile leaves lack abaxial trichomes and adult leaves are covered in abaxial trichomes from proximal to distal ends (Chien and Sussex, 1996; Telfer et al., 1997; Willmann and Poethig, 2011). Additionally, juvenile leaves tend to be flat and round with smooth margins, while adult leaves are curled and spatulate with serrated margins (Poethig, 2003, 2010). rdr6 loss-of-function mutants produce abaxial trichomes 1 – 2 leaves sooner than wild type, and their early leaves are more elongated and curled, phenotypes suggesting precocious vegetative phase change (Peragine et al., 2004; Vazquez et al., 2004) (see Figures 5A and 5B). This set of phenotypes was also seen in loss-of-function mutants of zip/ago7 (zippy/argonaute7) (Hunter et al., 2003), sgs3 (suppressor of gene silencing3) (Peragine et al., 2004; Vazquez et al., 2004), and dcl4 (dicer-like4) (Yoshikawa et al., 2005), all genes involved in post-transcriptional gene silencing pathways or paralogs of known silencing components. At the time, the only RNA silencing mutants known to have developmental phenotypes were involved in miRNA or transcriptional gene silencing, and RDR6 does not act in these pathways. Genetic and phenotypic analyses suggested that RDR6, ZIP, SGS3, and DCL4 act in a novel, silencing pathway that targets endogenous genes, which turned out to be the ta-siRNA biogenesis pathway, as described earlier.

The implication of this work was that the precocious developmental phenotype of these loss-of-function silencing mutants was caused by the inappropriate up-regulation of one or more targets of ta-siRNAs. In an attempt to find this target(s), microarrays were used to probe the transcriptomes of two-week-old wild-type, rdr6, sgs3, and zip plants (Peragine et al., 2004). The two most highly up-regulated genes in all three mutants were the transcription factors ETT/ARF3 and ARF4 (Peragine et al., 2004), and subsequently the ta-siRNA-producing locus TAS3 was found to produce ta-siRNAs called tasiARFs that direct the silencing of these transcripts (Allen et al., 2005; Williams et al., 2005). The tasiARF pathway is initiated by the miRNA miR390, which associates with ZIP/AGO7-RISC to direct the cleavage of TAS3. RDR6, SGS3, and DCL4 allow for the production of tasiARFs from the cleavage products, and the tasiARFs then target ETT and ARF4 for degradation (Allen et al., 2005; Williams et al., 2005). This regulatory module was confirmed by the observations that mutations in ETT or ARF4 were able to partially suppress the early vegetative phase change phenotype of rdr6, sgs3, and zip, and overexpression of ETT in transgenic plants was able to phenocopy these mutants (Fahlgren et al., 2006; Hunter et al., 2006). In retrospect, it was important to perform the microarray experiment with all three mutants because, while RDR6 and SGS3 are important for the biogenesis of ta-siRNAs from TAS1, TAS2, TAS3, and TAS4, ZIP is required only for those from TAS3, and a common target of all three proteins was most likely responsible for the phenotypes (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005; Williams et al., 2005; Adenot et al., 2006; Fahlgren et al., 2006; Hunter et al., 2006; Rajagopalan et al., 2006).

Interestingly, the SPL transcription factor SPL3, was also up-regulated in rdr6, sgs3, and zip (Peragine et al., 2004). SPL3 and at least several others of the 16 SPL proteins in Arabidopsis are important positive effectors of vegetative phase change and, unlike ETT and ARF4, their expression temporally increases during development (Cardon et al., 1997; Cardon et al., 1999; Hunter et al., 2006; Wu and Poethig, 2006; Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009). Currently, there is no explanation for the up-regulation of SPL3 in these ta-siRNA silencing pathway mutants, as ta-siRNAs are not known to regulate SPL genes. However, the misexpression of SPL3 may also contribute to the early vegetative phase change phenotype of rdr6.

The function of RDR6 in leaf adaxial-abaxial polarity

In addition to affecting developmental timing, the misexpression of ETT and ARF4 in rdr6 mutants also results in adaxial-abaxial polarity defects in leaves. The specification of three developmental axes—proximodistal, lateral-medial, and adaxial-abaxial—is required for normal leaf formation. Of these axes, a proper balance between adaxial and abaxial programs is necessary for the laminar nature of leaves, the polar differentiation of mesophyll (palisade versus spongy) and vascular cells (xylem versus phloem), and for the greater relative abundance of stomata on the abaxial surface (reviewed in Chitwood et al., 2007; Pulido and Laufs, 2010; Szakonyi et al., 2010). Adaxial-abaxial polarity is controlled in large part by the differential expression of specific transcription factors and smRNAs that intricately regulate each other’s expression. For instance, HD-ZIPIII family transcription

![Figure 5. Some developmental phenotypes of rdr6.](image-url)

Wild-type Col-0, rdr2-1, and rdr1-1 are included for comparison. All plants were grown under long-day conditions. (A) and (B) rdr6-11 plants display early vegetative phase change. (A) rdr6-11 mutant leaves are more downwardly curled, as seen in these four-week-old plants. (B) rdr6-11 leaves are more elongated, and the first leaf with abaxial trichomes appears earlier. The first three leaves of representative plants are shown. Numbers designate the first leaf with abaxial trichomes +/- standard deviation. (C) rdr6-11 flowers display stigma exsertion, which reduces self-fertility. Scale bar = 1 mm.
factors (including REV, PHB, and PHV) are expressed in the adaxial domain and act as adaxial determinants (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003), while KANADI (KAN1, KAN2, and KAN3) and YABBY family (FILAMENTOUS FLOWER/FIL, YAB2, and YAB3) transcription factors, as well as the miRNAs that target REV, PHB, and PHV (MIR165 and MIR166), are expressed in the abaxial domain and direct abaxial identity (Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; Emery et al., 2003; Eshed et al., 2004; Kidner and Martienssen, 2004; Yao et al., 2009). Thus, the interrelated regulation of these genes, altering the expression level or pattern of one or more of them in transgenic or mutant plants can profoundly alter leaf polarity. Such changes result in phenotypes that range from leaf curling and twisting to the formation of pin-like, radialized projections or whole organs due to the partial or complete absence, respectively, of one regulatory program (McConnell and Barton, 1998; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001; Emery et al., 2003; Eshed et al., 2004). These projections and some of the other phenotypes are due to patches of or the complete conversion of cells on the abaxial surface to an adaxial identity, or vice versa.

Recently, it was shown that the differential expression and localization of TAS3, tasiARFs, and ARF3/4 in leaf primordia are also important for establishing the adaxial/abaxial balance. While MIR390 is expressed throughout the lamina, ZIP/AGO7 and TAS3 are specifically found in the outermost two cell layers of the adaxial surface, including the epidermis (Garcia et al., 2006; Chitwood et al., 2009; Schwab et al., 2009). tasiARFs are synthesized in these cells, but then move, forming a gradient into the abaxial surface (Chitwood et al., 2009; Schwab et al., 2009). The result is that the expression of ARF3 and ARF4 is restricted to the abaxial domain (Pekker et al., 2005; Chitwood et al., 2009). The misexpression of ARF3/ARF4 in the adaxial surface in rdr6, zip/ago7, dcl4, and tas3 mutant leaves likely causes the downward leaf curling manifested in these plants. None of these mutants have other polarity-associated phenotypes, however (Peragine et al., 2004; Vazquez et al., 2004; Yosikawa et al., 2005; Adenot et al., 2006), most likely due to partial redundancy between the ARF and KAN proteins. This hypothesis is supported by the observations that gross, systemic overexpression of ARF3 results in abaxialized leaves that are downwardly curled, twisted, and lobed with ectopic, radialized leaf primordia on the abaxial surface (Fahlgren et al., 2006; Hunter et al., 2006). Additionally, arf3 arf4 double mutants show a complementary, adaxialized phenotype, like that seen in kan1 kan2 — upward leaf curling and ectopic, radialized abaxial outgrowths (Eshed et al., 2001; Pekker et al., 2005). These phenotypes associated with the misexpression of ARF3 and/or ARF4 are due to the ability of ARF3 and ARF4 to up-regulate YABBY gene expression in parallel to KAN proteins, thereby promoting abaxial cell fate (Garcia et al., 2006). Thus, RDR6 promotes adaxial identity and represses abaxial identity through its role in the biogenesis of tasiARFs in the adaxial domain.

The function of RDR6 in lateral root production

The site of a new lateral root is determined by the presence of a polar auxin maximum (peak of auxin accumulation) within the pericycle, the layer of cells just below the endodermis (De Smet et al., 2007; Dubrovsky et al., 2008). Lateral root primordia are formed from transverse division of the pericycle at this site followed by 2 – 4 periclinal divisions. This results in 3 – 5 layers of isodiametric cells that consequently form a lateral root meristem (Laskowski et al., 1995). Thus, three major steps characterize lateral root production: the designation of the site of a new lateral root by auxin activation of pericycle cells, the formation of a primordium made up of equally-sized cells, and the establishment of a meristem that will give rise to all future cells of the lateral root.

Recent evidence has implicated RDR6, also via its role in the biogenesis of TAS3-derived ta-siRNAs, in the elongation of lateral roots, possibly by promoting the maintenance of the lateral root meristem. rdr6 mutants have significantly shorter lateral roots than wild-type plants, though the total number and density of lateral roots are not different, and the primary root appears normal (Marin et al., 2010). Other mutants that have reduced levels of tasiARFs (dcl4, mir390a, and tas3a) have a similar phenotype. Consistent with these findings, overexpressing TAS3, with the concomitant increase in tasiARF abundance, or down-regulating tasiARF targets ARF2, ARF3, and ARF4 using an artificial miRNA resulted in longer lateral roots. The slower progression of lateral root growth in rdr6, dcl4, mir390a and tas3a may be due to a smaller lateral root meristem in these mutants. Thus, TAS3 probably normally functions to reduce the expression levels of ARF2, ARF3, and ARF4, whose encoded proteins likely delay or reduce the activity of the emerging lateral root meristem (Marin et al., 2010). While the reduced expression of these genes in a TAS3 overexpression line results in longer roots, it does not cause an enlarged meristem (Marin et al., 2010), however, probably because the levels of ARF2, ARF3, and ARF4 in wild-type plants are already below the necessary threshold (Marin et al., 2010; Yoon et al., 2010).

It is noteworthy that a delicate balance of auxin, MIR390a, RDR6, TAS3a, and ARF2/3/4 controls the process of lateral root elongation, which is partially determined by their expression domains. For example, MIR390a accumulates in the pericycle and mesenchymal xylem cells prior to the appearance of an auxin maximum, as well as at the base of emerging lateral roots (Marin et al., 2010; Yoon et al., 2010). However, the presence of MIR390a is not required for lateral root initiation, as the numbers of lateral roots did not change in the MIR390a mutant (Marin et al., 2010). At very early stages of lateral root elongation, the auxin maximum and MIR390a expression domain are coincident, but over time become distinct. Thus, it is not surprising that auxin can induce the expression of MIR390a, likely by indirect means, as cycloheximide can block this effect (Marin et al., 2010). TAS3 is expressed throughout the root in the undifferentiated parenchyma cells of the stele, overlapping with MIR390a at sites of lateral root emergence. Interestingly, tasiARFs, like mir390, are found throughout the emerging lateral root, showing that they can act non-cell autonomously in roots, as they also do in leaves (Chitwood et al., 2009; Schwab et al., 2009). This movement reduces ARF2, ARF3, and ARF4 expression in the emerging lateral root, but these ARFs also impact the expression of MIR390a and RDR6. Specifically, ARF3 enhances the expression of MIR390a, while ARF4 reduces the expression of both MIR390a and RDR6 (Marin et al., 2010; Yoon et al., 2010). The positive regulation by ARF3 may explain why the abundance of MIR390a is induced by auxin treatment (Marin et al., 2010). Thus, RDR6 is necessary for lateral root elongation also due to its role in the biogenesis of tasiARFs.
RDR6 and anthocyanin production

Beyond TAS3, the developmental roles of RDR6 have only been associated with one other trans-acting siRNA-producing locus—TAS4, which produces ta-siRNAs that target the transcripts of the MYB transcription factors PAP1/MYB75, PAP2/MYB90, and MYB113 (Rajagopalan et al., 2006; Hsieh et al., 2009). The products of these genes induce the expression of many biochemical enzymes in the anthocyanin biosynthetic pathway (Borevitz et al., 2000; Tohge et al., 2005; Gonzalez et al., 2008). Interestingly, increased anthocyanin production is a common response and an important survival mechanism to multiple stresses, including nutrient deficiency (Steward et al., 2001; Scheible et al., 2004; Missen et al., 2005; Bi et al., 2007; Lea et al., 2007; Morcuende et al., 2007; Peng et al., 2007; Lillo et al., 2008; Hsieh et al., 2009; Olsen et al., 2009). For example, in the absence of phosphate or nitrogen in the growth medium, the expression of PAP1, PAP2, and MYB113 is significantly increased, with a concomitant rise in anthocyanin levels (Scheible et al., 2004; Missen et al., 2005; Bi et al., 2007; Lea et al., 2007; Morcuende et al., 2007; Peng et al., 2007; Hsieh et al., 2009; Olsen et al., 2009). Recently, it was demonstrated that tas4 mutants accumulate higher than normal levels of anthocyanin when plants are grown under full nutrition with even higher levels under phosphate or nitrogen deficiency. The main role of TAS4 may be to prevent the overaccumulation of anthocyanin under stress because TAS4-derived ta-siRNAs increase in abundance under phosphate or nitrogen deficiency due to an up-regulation of the levels of the miRNA that initiates their production (miR828) (Hsieh et al., 2009). While there is no published report of increased anthocyanin levels in rdr6 loss-of-function mutants, the anticipated absence of TAS4-derived ta-siRNAs when RDR6 function is lacking should result in phenotypes similar to tas4.

RDRs and plant reproduction

When rdr6 was first identified as a regulator of developmental timing, it was observed that this mutant also manifested floral defects, including stigma exertion, a split septum, and ectopic stigmatic tissue on the inner ovary wall at the apical end of the carpels (Peragine et al., 2004), reminiscent of phenotypes reported for zip/ago7 mutants (Hunter et al., 2003). The split septum phenotype was due to the overexpression of ARF4 in rdr6 and zip/ago7 (Hunter et al., 2006). Since then, RDRs have been shown to regulate reproductive development in Arabidopsis at multiple steps, including female gametophyte development, the maternal-to-zygotic transition, self-fertility, and double fertilization (Tantikanjana et al., 2009; Olmedo-Monfil et al., 2010; Ron et al., 2010; Autran et al., 2011). We will consider each of these in turn.

Female gametophyte development begins with the specification of a sporophytic cell as a megasporocyte mother cell. This cell undergoes meiosis to yield four haploid cells, three of which degenerate, while one becomes the functional megaspore. The megaspore then undergoes three rounds of mitosis to yield a seven cell, eight nuclei female gametophyte, with one of the cells being the egg (reviewed in Drews and Yadegari, 2002).

In a high frequency of rdr6 and rdr2 mutant ovules, there are multiple abnormally enlarged cells instead of a single distinctive megasporocyte mother cell, but only one undergoes meiosis (Olmedo-Monfil et al., 2010). In fact, a marker that is normally expressed only in the megaspore and mature female gametophyte was found in four or more cells of mutant ovules. These results suggest that some somatically-derived cells have a haploid megasporocyte identity in these mutants. Thus, it is not surprising that over 40% of the time this inappropriate identity results in two complete female gametophytes. This developmental anomaly was not associated with reduced fertility from ovule or seed abortion, however, because only one of the gametophytes develops into an embryo. These results suggest that RDR6 and RDR2 are required for the biogenesis of smRNAs that restrict the identity of somatic cells in the ovule, such that only one attains a megasporocyte mother cell identity and no somatic cells act as megaspores (Olmedo-Monfil et al., 2010).

Similar results were seen in sgs3 but not dcl1 or dcl4, implying that miRNAs and ta-siRNAs are not responsible for the phenotypes. In addition, mutants of several other transcripational silencing pathway components (ago9, dcl3, and the double mutant nrpd1a nrpd1b, deficient in both Pol IV and V) had the same phenotypes. This is intriguing because AGO9 protein is not found in the megasporocyte mother cell or the female gametophyte but is present in the surrounding somatic companion cells, suggesting that smRNAs made in these cells can move into the megasporocyte mother cell, megasporocytes, and/or female gametophyte. In summary, these findings have led to a model where Pol IV and/or V, RDR2, RDR6, and DCL3 produce smRNAs that are mobilized by incorporation into AGO9-RISC. These AGO9-bound smRNAs then enter the surrounding somatic cells, but not the female gametophyte precursors, to suppress target transcripts and/or loci. In such a model, RDR6 and SGS3 act to amplify the signal in the somatic cells (Olmedo-Monfil et al., 2010). Future studies will be necessary to test this model and identify the specific smRNAs and their targets.

In addition to its role in producing mobile smRNAs that regulate female gametophyte development, RDR2, through its function in RdDM, is also required to transcriptionally silence zygotic loci during the early stages of Arabidopsis development, allowing maternal transcripts (originally present in the egg cell) to dominate during this time (Autran et al., 2011). Maternal control of the embryonic transcriptome has been observed in many eukaryotes, including mammals, but the involvement of epigenetic pathways in this process had not been observed for any organism previously (Zilberman, 2011). It was recently found that RDR2-dependent RdDM and heterochromatin formation are necessary for maternal dominance of the early zygotic transcriptome in Arabidopsis. In fact, if maternal RDR2 is non-functional, paternally-inherited genes that are normally lowly expressed or absent in 2 – 4 cell embryos are activated in this early developmental stage instead of later (globular stage) in embryogenesis (Autran et al., 2011). Preliminary evidence has led to an intriguing hypothesis that this transcripational silencing of zygotic loci early in embryonic development is likely a consequence of RDR2-dependent production of 24 nt smRNAs that can then direct RdDM-mediated transcriptional silencing (Autran et al., 2011). Interestingly, these RDR2-dependent 24 nt smRNAs may actually be produced during female gametogenesis. In support of this theory, it was recently found that AGO9 is associated with numerous 24 nt smRNAs that are produced from protein-coding genes in developing Arabidopsis ovules (Olmedo-Monfil et al., 2010). Because they are 24 nt...
in length, these smRNAs can likely direct RdDM-mediated transcriptional silencing to protein-coding genes, thereby allowing the transcriptome contributions specifically from the mother to dominate the early embryonic transcriptome. Future studies will be necessary to test this model and to determine if the smRNAs that negatively regulate zygotic loci in early Arabidopsis embryogenesis are specifically produced in maternal reproductive tissues.

RDR6 has also been implicated in self-fertility. Unlike most Brassica species, Arabidopsis is self-compatible, meaning that pollen can fertilize eggs from the same plant, but it evolved from a self-incompatible ancestor. Self-incompatibility in Brassicas is controlled by the S-locus, which consists of a receptor kinase (SRK) expressed in the stigma and a tightly-linked, coevolving, cysteine-rich protein ligand (SRC, also called SP11) localized to the pollen coat, also a sporophytic tissue (Stein et al., 1991; Schofer et al., 1999; Takasaki et al., 2000; Takayama et al., 2000). The S-locus is highly polymorphic, and an SRK can be activated upon directly binding only the SRC from the same locus (Takayama et al., 2001; Kachroo et al., 2002). Upon this activation, a signaling cascade is initiated that suppresses pollen tube growth. Additionally, self-incompatible species are sometimes associated with differences in floral morphology that further promote out-crossing (Barrett, 2010). One of these is an increased distance between the stigma and the anthers, with the stigma above the anthers, called stigma exertion.

_A. lyrata_, which is evolutionarily separated from _A. thaliana_ by five million years, is self-incompatible (Koch et al., 2000). Because their most recent common ancestor was likely self-incompatible, it is not surprising that several accessions of _A. thaliana_ can be made self-incompatible by transformation with a functional S-locus (AlSRKb-AlSCRb) from _A. lyrata_, suggesting that the self-compatibility of these accessions was due to a loss of a functional S-locus (Nasrallah et al., 2002; Nasrallah et al., 2004; Boggs et al., 2009b; Boggs et al., 2009a). Interestingly, expressing AlSRKb-AlSCRb in the Columbia accession results in a developmentally transient self-incompatibility that breaks down in older flowers (Nasrallah et al., 2002). This trait allowed for the screening of enhancers of self-incompatibility (Tantikanjana et al., 2009). One such enhancer was a loss-of-function mutation in RDR6. While AlSRKb-AlSCRb plants have morphologically normal flowers, rdr6 AlSRKb-AlSCRb plants displayed stigma exertion, in addition to having a stronger self-incompatibility phenotype and producing less seed. rdr6 is not self-incompatible on its own, but it does have stigma exertion (Figure 5C), though this phenotype is further exaggerated in the AlSRKb-AlSCRb background. The exaggerated stigma exertion phenotype requires a kinase-active AlSRKb, but it does not require AlSCRb. Similar phenotypes were also seen in double mutants between AlSRKb-AlSCRb and zip/ago7 but not rdr2, suggesting the involvement of ta-siRNAs. Because the primary guide for ZIP/AGO7 is miR390, it is possible that the misregulation of the miR390-TAS3-ARF2/3 pathway in rdr6 is responsible for the self-incompatibility and stigma exertion phenotypes, though this remains to be tested. Together, these results indicate that RDR6 provides a molecular basis for the co-evolution of self-incompatibility and stigma exertion in out-crossing species (Tantikanjana et al., 2009).

The last role of RDRs in reproductive development that we will consider is in double fertilization, a unique feature of angiosperm seed development. To allow for double fertilization, the angiosperm male gametophyte consists of three cells—two sperm cells and a vegetative cell that forms the pollen tube. When the pollen tube reaches the ovule, one of the two sperm cells fertilizes the egg, yielding a zygote that will go on to form the embryo. The second sperm fuses with the two polar nuclei of the central cell to yield a triploid cell that will develop into the endosperm. In Arabidopsis, the use of a homozygous loss-of-function mutant of KOKPELLI (KPL) as the pollen parent results in 70% reduced seed set due to a high proportion of single fertilization events, where either the egg cell or the central cell is fertilized but not both. This single fertilization resulted in undeveloped ovules and aborted seeds. Conversely, normal seed set is observed when pollinating _kpl_ mutants with wild-type pollen, demonstrating a pollen-specific effect (Ron et al., 2010). _KPL_ is expressed in the sperm nuclei but not the vegetative cell, which is the inverse pattern as _ARI-ADNE14_ (ARI14), a gene that encodes a putative ubiquitin E3 ligase and is found in antisense orientation to _KPL_ with 82 bp between their stop codons. Interestingly, the _ARI14_ promoter is active in all three cells of the male gametophyte, but its expression is post-transcriptionally down-regulated in the sperm nuclei by a nat-siRNA processed from the region of complementarity between _KPL_ and _ARI14_. In fact, the _kpl_ mutant phenotype is a consequence of _ARI14_ misexpression in the sperm nuclei. This was confirmed by overexpressing an siRNA-insensitive form of _ARI14_ in sperm nuclei, which phenocopied _kpl_ mutant plants. The authors report that mutants in _DCL1, HEN1, HYL1, SGS3_, and _RDR2_ (but not _RDR6, DCL2, DCL3_, or _DCL4_) have increased expression of _ARI14_ in one-day-old open flowers, suggesting that these genes are involved in the biogenesis of the nat-siRNA from _KPL_ (Ron et al., 2010). These results are consistent with the biogenesis of other known nat-siRNAs, except that in the two other cases _RDR6_ was required rather than _RDR2_ (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). Further study will be necessary to determine the basis for the requirement of different RDRs in nat-siRNA biogenesis. It is of note that the regulation of _ARI14_ by _KPL_ seems to be evolutionarily recent. Although _ARI_ proteins are conserved throughout eukaryotes, a _KPL_ locus is found in some, but not all, Brassicaceae family members, and _KPL_ is not always in close enough proximity to the orthologs of _ARI14_ to drive formation of nat-siRNAs (Ron et al., 2010). Thus, plants must use a variety of molecular mechanisms to ensure double fertilization, one of which involves RDR proteins.

**Conservation of RDR developmental modules in plants**

Some of the developmental roles of _RDR6_ discussed above are conserved in other plants. For instance, knockouts of _RDR6_ orthologs in the moss _Physcomitrella patens_ showed delayed transition from juvenile to mature stages of development (Talmor-Neiman et al., 2006). The same miRNA — miR390 — is involved in this pathway as in Arabidopsis, but the moss TAS genes do not have any homology outside of the miRNA-binding site (Arazi et al., 2005; Axtell et al., 2006; Talmor-Neiman et al., 2006; Axtell et al., 2007). The specific _RDR6_-dependent smRNAs and targets involved in this case have not been demonstrated. However, it is worth noting that ta-siRNAs targeting ARF genes are also present in _P. patens_ (Axtell et al., 2007), indicating a degree of conservation for RDR targets in land plants.
The miR390-TAS3-ARF pathway components appear to be conserved in all seed plants (Axtell et al., 2006), and the module’s specific role in leaf polarity has been demonstrated in maize and rice (Liu et al., 2007; Nogueira et al., 2007; Nogueira et al., 2009; Douglas et al., 2010; Wang et al., 2010a). Furthermore, the importance of this pathway to leaf polarity seems to be greater in these species than Arabidopsis based on the phenotypes of the loss-of-function mutations in the orthologs. For example, mutants in SGS3 and ZIP/AGO7 in maize — leafbladeless1 (lbf1) and ragged seedling2 (rgd2), respectively — and RDR6 (shootless2, sh2) and DCL4 in rice show strong polarity phenotypes compared to their Arabidopsis counterparts (Timmermans et al., 1998; Hunter et al., 2003; Satoh et al., 2003; Peragine et al., 2004; Vazquez et al., 2004; Henderson et al., 2005; Yoshikawa et al., 2005; Garcia et al., 2006; Hunter et al., 2006; Liu et al., 2007; Nagasaki et al., 2007). As seen for Arabidopsis, tasiARFs in maize form a gradient from the adaxial to the abaxial surface, and ARF genes are expressed specifically in the abaxial surface (Nogueira et al., 2007). However, the tasiARF distribution in maize is determined not by the expression domain of TAS3, but by the expression of MIR390. MIR390 is expressed in the adaxial epidermis, but the mature miRNA is also found in the underlying adaxial cell layers, suggesting possible movement (Nogueira et al., 2009). Also like Arabidopsis, the tasiARFs promote adaxial identity in part by restricting the expression or abundance of miR166, an abaxial determinant (Li et al., 2005; Nogueira et al., 2007; Douglas et al., 2010). This was demonstrated by the finding that mature miR166 inappropriately accumulates in the adaxial domain in maize lbf1 and rgd2 mutants (Nogueira et al., 2007; Douglas et al., 2010). Furthermore, overexpression of a tasiARF in rice resulted in the down-regulation of OsARF3 with a concomitant decrease in miR166 (Wang et al., 2010a). Thus, while the mechanism controlling the adaxial-specific expression of tasiARFs may differ between Arabidopsis and monocots, their downstream effects on ARF3 and MIIR166 expression are conserved, further supporting the importance of this pathway.

**RDRs in Pathogen Resistance**

**RDRs in antiviral resistance**

smRNA-mediated RNA silencing is believed to have evolved as a defense against invading nucleic acids such as transposons and viruses (Matzke and Matzke, 1995; Baulcombe and English, 1996; Ratcliffe et al., 1997; Al-Kaff et al., 1998). In support of this hypothesis, plants defective in components of the silencing machinery are more susceptible to viral infection (Mourrain et al., 2000; Morel et al., 2002; Boutet et al., 2003; Yu et al., 2003; Muangsang et al., 2004; Schwach et al., 2005; Adenot et al., 2006). For instance, loss-of-function mutations in Arabidopsis RDR1 (Yu et al., 2003; Diaz-Pendon et al., 2007; Donaire et al., 2008; Qi et al., 2009; Garcia-Ruiz et al., 2010; Wang et al., 2010b), RDR2 (Donaire et al., 2008; Garcia-Ruiz et al., 2010), or RDR6 (Mourrain et al., 2000; Muangsang et al., 2004; Xie et al., 2004; Adenot et al., 2006; Diaz-Pendon et al., 2007; Qi et al., 2009; Wang et al., 2010b) have an increased susceptibility to a variety of plant viruses, increased accumulation of viral RNA, and/or a decreased accumulation of viral-derived siRNAs (vsiRNAs). In addition, RDR1 expression is induced by TMV infection and salicylic acid treatment (Yu et al., 2003; Xie et al., 2004; Diaz-Pendon et al., 2007). The up-regulation of RDR1 in response to salicylic acid is significant because SA is important for systemic acquired resistance against subsequent viral infections and spreading (Gaffney et al., 1993; Naylor et al., 1998; Murphy and Carr, 2002). Additionally, RDR1 and RDR6 orthologs in other species have also been implicated in RNA silencing of viruses (Gaffney et al., 1993; Naylor et al., 1998; Murphy and Carr, 2002; Qu et al., 2005; Schwach et al., 2005; Pandey et al., 2008). Viral and/or salicylic acid-inducible RDR1 orthologs have also been reported in Nicotiana spp., Medicago truncatula, and Zea mays (Yang et al., 2004; Alamillo et al., 2006; He et al., 2010). In total, the conservation of the particular RDRs involved in viral silencing and the control of their expression emphasizes their importance to plant defense responses. The use of RNA silencing by plants as a viral defense mechanism is not surprising because the genetic material of the majority of plant viruses is ssRNA or dsRNA, and the ssRNA viruses frequently can form extensive stem-loop structures (Molnar et al., 2005; Ho et al., 2006; Donaire et al., 2009) and/or replicate via a dsRNA intermediate (Pogue et al., 1994; Buck, 1999).

In response to this host antiviral defense system, viruses have evolved genes whose products are capable of inhibiting RNA silencing. These proteins are known as viral RNA silencing suppressors, or VSRs, and have been identified in nearly all types of viruses infecting plants, as well as those attacking many other organisms. VSRs can suppress silencing through a variety of mechanisms, and thus target many different steps in the RNA silencing pathway (recently reviewed in Diaz-Pendon and Ding, 2008; Miotshwa et al., 2008a; Alvarado and Scholthof, 2009; Burgyan and Havelda, 2011).

Plant antiviral silencing has been visualized as a three-part process, which includes initiation, amplification, and systemic spread (Figure 6). For antiviral silencing to begin, a dsRNA trigger must be recognized by the plant. This dsRNA will then be used for generation of DCL-dependent primary vsiRNAs. For RNA viruses, vsiRNAs are generated primarily by DCL4, with DCL2 (largely in the absence of DCL4) and DCL3 playing lesser roles (Xie et al., 2004; Bouche et al., 2006; Deleris et al., 2006; Qu et al., 2008; Garcia-Ruiz et al., 2010), while all four DCLs are required for vsiRNAs from DNA viruses (Akbergenov et al., 2006; Blevins et al., 2006). Once primary vsiRNAs are made, they are loaded into AGO1 or AGO2 containing-RISC complexes, which target long viral transcripts for degradation (Morel et al., 2002; Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007; Pantaleo et al., 2007; Qu et al., 2008; Takeda et al., 2008; Harvey et al., 2011; Wang et al., 2011). This serves not only to destroy the virus and limit its infectivity (see systemic spread below), but also to generate large amounts of aberrant RNA that can be targeted by the endogenous RDRs. RDRs may generate secondary vsiRNAs, which are processed from regions of the virus that are distant from the sites targeted by primary vsiRNAs, a mechanism known as transitivity (Voinnet, 2008). The generation of RDR-dependent secondary vsiRNAs serves to increase the pool of functional anti-viral vsiRNAs and effectively amplify the silencing response. This places RDRs at the center of the second part of the plant response to viral infection—amplification (Figure 6).

Although RDRs are required for the biogenesis of secondary vsiRNAs (Diaz-Pendon et al., 2007; Donaire et al., 2008; Qi et al.,
Figure 6. A model of RDR function in Arabidopsis anti-viral defense.

The host silencing machinery recognizes virally-derived dsRNA structures, at which point DCL-dependent primary vsiRNAs are generated. This occurs in the initiation phase and requires the function of DCL4 and DCL2. Once primary vsiRNAs are made, they are loaded into RISC complexes most likely containing AGO1 or AGO2. These vsiRNA-loaded RISC complexes are capable of targeting long viral transcripts for degradation. This mechanism serves not only to destroy the virus and limit its spread, but also to produce large amounts of aberrant RNA that can be targeted by RDR1 and RDR6. These RDRs likely generate additional vsiRNAs by augmenting the level of existing dsRNA that can be subsequently processed by DCL4 and DCL2 to yield more vsiRNAs. The RDR-dependent vsiRNAs are secondary vsiRNAs and target viral regions that are distant from the sites of primary vsiRNA processing. RDR1 and RDR6 also work additively or cooperatively to inhibit viruses from systemically infecting the plant. Therefore, these RDRs are also required for the production of the mobile silencing signal that acts to immunize plants against systemic and secondary viral infections.

2009; Wang et al., 2010b; Wang et al., 2011), the members of this class of proteins that function in this process had remained unclear until recently. This is because deficiency of specific RDRs did not always correlate with enhanced viral accumulation (Qi et al., 2009). To address this conundrum, two groups (Garcia-Ruiz et al., 2010; Wang et al., 2010b) improved upon the well-established approach of using VSRs to study viral RNA silencing in plants. Specifically, one group (Garcia-Ruiz et al., 2010) infected wild-type Arabidopsis and a series of rdr mutants, including rdr1, rdr2, rdr6, and all double and triple mutant combinations, with...
either a wild-type Turnip Mosaic Virus (TuMV) that encodes an effective VSR termed Helper Component Proteinase (HC-Pro) or transgenic TuMV containing a nonfunctional HC-Pro (referred to here as TuMVΔHC). Wild-type TuMV has been demonstrated to readily infect Arabidopsis tissue, resulting in easily observable symptoms (Kasschau et al., 2003). Interestingly, while rdr1 and rdr6 mutants had previously been shown to have increased susceptibility to other viruses, it was found that challenging Arabidopsis with TuMV produced identical disease symptoms in wild-type plants and all of the rdr mutant plants (Garcia-Ruiz et al., 2010). By contrast, TuMVΔHC was unable to infect any of the genotypes except those that had the rdr1 mutation. Furthermore, the rdr1 single mutant and all double and triple mutants containing a defective RDR1 gene exhibited significantly reduced accumulation of vsiRNAs, indicating a role for this protein in viral resistance and vsiRNA accumulation. A similar study using Cucumber Mosaic Virus (CMV) and a VSR-defective version (CMVΔ2B) also revealed a role for RDR1, as well as RDR6, in viral silencing (Wang et al., 2010b). Specifically, when wild-type Arabidopsis plants were challenged with CMVΔ2B, they manifested no observable disease symptoms. Conversely, rdr1 rdr6 double mutant plants demonstrated increased disease symptoms that correlated with a significant increase in viral RNA and a concomitant decrease in vsiRNAs. Interestingly, for both TuMV and CMV a background level of vsiRNAs were observed in rdr1 rdr6 double mutants that are completely undetectable in plants that have lost the function of both Dicer-like proteins responsible for generating primary and secondary vsiRNAs during antiviral silencing (dcl2 dcl4). This suggests that the RDR1/6-independent vsiRNAs are primary vsiRNAs that are generated directly from viral replication products. Alternatively, these vsiRNAs could be the products of residual activity from another RDR protein. Taken together, these results indicate that RDR1 and RDR6 function specifically in the amplification but not the initiation of silencing of these viruses.

Once the silencing response to a viral challenge has been initiated, the plant sends a signal to the surrounding cells, initiating sequence-specific silencing against the invading and related viruses in distal tissues. This systemic spread of silencing eventually encompasses the entire plant, effectively providing a plant-wide immunization (Figure 6) (Ding and Voinnet, 2007; Motshwa et al., 2008b). The mobile silencing signal can move locally between cells through plasmodesmata, and systemically via a phloem-dependent mechanism (Dunoyer et al., 2005). It has been shown that silencing can initially spread ~10-15 cells and initiate silencing in these cells even in the absence of RDR6. However, movement beyond 10-15 cells requires RDR6 (Voinnet, 2005). This suggests that secondary (RDR-dependent) but not primary (RDR-independent) vsiRNAs are required for the systemic spread of anti-viral silencing. This idea is supported by the work done with the TuMVΔHC virus (Garcia-Ruiz et al., 2010). Specifically, in wild-type plants the virus was unable to move beyond the initial point of infection, indicating that the silencing signal was able to immunize surrounding cells against the systemic spread of the virus. However, in rdr1 and rdr6 mutant plants the virus was able to move systemically from the point of initial infection, revealing that RDR1 and RDR6 work coordinately to prevent viral spread (Garcia-Ruiz et al., 2010). Therefore, RDRs are important for both the amplification and spread of antiviral silencing in plants.

RDRs in antibacterial resistance

While the role of RDRs in antiviral defense is well documented, these proteins also function in the production of siRNAs that are required for defense against other plant pathogens (Katyar-Agarwal et al., 2006; Hewezi et al., 2008). RDR6 has been shown to be important for the production of multiple bacterially-induced siRNAs in Arabidopsis (Katyar-Agarwal et al., 2006; Katyar-Agarwal et al., 2007). For example, in response to infection by the pathogenic bacterium Pseudomonas syringae pv tomato (hereafter referred to as Pst) carrying the avirulence gene avrRpt2, an RDR6-dependent nat-siRNA (nat-siRNAATGB2) is produced from a region of overlap between the 3′ UTRs of ATGB2 and PPRL, a negative regulator of RPS2-dependent Pst defense responses in the host. Within this natural antisense gene pair, it is ATGB2 whose expression is induced in plants infected by this specific Pst strain. The induction of ATGB2 occurs when the bacterial effector protein avrRpt2 is indirectly recognized by its specific R-gene receptor RPS2. Pathogen effector/R-gene interaction initiates a cascade of defense responses that includes induction of ATGB2 expression. The increase in ATGB2 leads to the subsequent accumulation of nat-siRNAATGB2 in an RDR6-dependent manner. nat-siRNAATGB2 is then incorporated into RISC and mediates a decrease in PPRL (Katyar-Agarwal et al., 2006). Therefore, RDRs are also necessary to mediate the plant response to some bacterial pathogens.

RDRs in antinematode resistance

It was recently demonstrated that ta-siRNAs are also likely important for plant responses to pathogenic organisms. Several RDR6-dependent ta-siRNAs were found to be differentially regulated upon attack by the cyst-forming nematode Heterodera schachtii, and mutants of rdr6 and rdr1 were less susceptible to infection (Hewezi et al., 2008). This result suggests that an unidentified target of RDR-dependent silencing increases resistance to H. schachtii. In summary, these findings suggest that RDR-dependent siRNA pathways in Arabidopsis play a widespread role in plant defenses against an array of different pathogens.

RDRs in ABIOTIC STRESS RESPONSE

Given the extent to which the RDR-mediated small RNA pathways are involved in biotic stress responses, it is not surprising that they also have a role in plant response to abiotic stresses. As discussed earlier, the expression of certain RDRs is affected by abiotic stresses in Arabidopsis (Figure 4C) and in other species (Yang et al., 2008; Madsen et al., 2009; Yang et al., 2010). Further, the first identified nat-siRNAs were identified in Arabidopsis as salt-stress responsive smRNAs (Borsani et al., 2005). In Arabidopsis there is a cis natural anti-sense gene pair that consists of P5CDH, which encodes Δ1-pyrroline-5-carboxylate dehydrogenase, and SRO5, a gene of unknown function that is only expressed under high salt conditions. Co-expression of these two genes results in the formation of a partially overlapping dsRNA molecule consisting of the very 3′ ends of both transcripts. Subsequently, this dsRNA acts as a substrate for small interfering RNA (siRNA) biogenesis. These siRNAs direct initial cleavage
of P5CDH, thus resulting in an RNA molecule that can act as a substrate for dsRNA synthesis by RDR6. The dsRNA molecules produced by RDR6 are processed by DCL1 into 21 nt siRNAs that are incorporated into RISC. Ultimately, this antisense pair of transcripts results in the down-regulation of P5CDH levels and improved salt tolerance (Borsani et al., 2005). Thus, RDRs also regulate abiotic stress responses in Arabidopsis. It is interesting to note that close to 9% of all Arabidopsis genes have the potential to be expressed as an overlapping antisense gene pair, suggesting the potential involvement of nat-siRNAs in many environmental stress responses (Jen et al., 2005; Wang et al., 2005).

In addition to the role of RDR6-dependent nat-siRNAs in the regulation of plant environmental stress responses, other RDR-dependent siRNAs appear to do so, as well. For instance, RDR6-dependent ta-siRNAs derived from TASt transcripts are reduced in cold treated plants, but this is not true for the miRNAs and other classes of smRNAs tested (Kume et al., 2010). This is likely because the target genes of these ta-siRNAs are required for regulating the response of Arabidopsis plants to cold temperatures. Additionally, it was demonstrated that other RDR6-dependent ta-siRNAs are responsive to hypoxia stress. In this case, it is likely that target RNA regulation by these smRNAs is necessary for plant responses to growth in low oxygen conditions (Moldovan et al., 2010). Recently, it was found that both RDR2 and RDR6 are also required for the correct response to genotoxic stress in Arabidopsis (Yao et al., 2010). One example of this type of stress is DNA damage that is induced by exposure to ultraviolet (UV) light (Li et al., 2002). In Arabidopsis, it was specifically observed that rdr2 mutant plants are more resistant to the DNA damaging agent methyl methane sulfonate (MMS) than wild-type plants, while the opposite is true for rdr6 mutant plants (Yao et al., 2010). Taken together, these results indicate that RDR proteins and multiple classes of siRNAs regulate the correct plant response to genotoxic stresses. Overall, it is evident that RDRs are necessary for proper responses to numerous environmental stresses in Arabidopsis, but there is still very little known about the functions of these proteins during such responses.

CONCLUSION

In summary, it is clear that plant RDRs play significant roles in Arabidopsis development and environmental responses through their functions in multiple smRNA-mediated RNA silencing pathways. However, our understanding of the full impact of these proteins on plant biology and endogenous RNA silencing pathways is still quite lacking. For instance, the endogenous functions of at least four Arabidopsis RDRs (RDR1, 3, 4, and 5) are almost entirely unknown (Figure 1). Furthermore, although it is clear that RDRs are important for biotic and abiotic stress responses, the mechanistic details of how these proteins function in such processes remain mostly unclear. To begin to fill these gaps in our knowledge of RDRs, a focus on identifying endogenous RDR1, 3, 4, and 5-dependent siRNAs and dsRNAs will be necessary, as has been done for RDR6 in flower buds (Zheng et al., 2010). Additionally, assaying for the effects of all six Arabidopsis RDRs on plant responses to a full panel of known pathogens and abiotic stress responses will allow us to determine how many of these processes require the function of RDRs. In conclusion, RDRs, proteins once thought to be required solely for silencing exogenous dsRNA, are also pivotal for an array of important endogenous processes in plants, many of which likely remain to be discovered.

ACKNOWLEDGEMENTS

This work was funded by NSF Career Award MCB-1053846 to BDG.

REFERENCES


stress and putative nitrogen regulatory components in Arabidopsis. BMC Genomics 8: 281.


Arabidopsis RNA-dependent RNA Polymerases 17 of 20


Satoh, N., Itoh, J., and Nagato, Y. (2003). The SHOOTLESS2 and SHOOTLESS1 genes are involved in both initiation and maintenance of the shoot apical meristem through regulating the number of indeterminate cells. Genetics 164: 335-346.
493-500.


