The Functions of RNA-Dependent RNA Polymerases in Arabidopsis

Authors: Willmann, Matthew R., Endres, Matthew W., Cook, Rebecca T., and Gregory, Brian D.

Source: The Arabidopsis Book, 2011(9)

Published By: The American Society of Plant Biologists

URL: https://doi.org/10.1199/tab.0146

First published on July 31, 2011: e0146. doi: 10.1199/tab.0146

The Functions of RNA-Dependent RNA Polymerases in Arabidopsis

Matthew R. Willmann[a,b], Matthew W. Endres[a,b], Rebecca T. Cook[a], and Brian D. Gregory[a,b,c,1]

[a]Department of Biology, [b]PENN Genome Frontiers Institute, [c]Genomics and Computational Biology Graduate Program
University of Pennsylvania, Philadelphia, PA 19104, USA
[1]Address correspondence to bdgregor@sas.upenn.edu

One recently identified mechanism that regulates mRNA abundance is RNA silencing, and pioneering work in *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 distinct 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 *Caenorhabditis elegans*, but are absent in *Drosophila*, mice, and humans. There are three major clades of

eukaryotic *RDR*s — *RDR*α, *RDR*β, and *RDR*γ — 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 *RDR*α genes have been sequenced from all three kingdoms, *RDR*β has only been found in animals and fungi and *RDR*γ only in plants and fungi. Thus, *RDR*β was likely lost in the plant lineage, while *RDR*γ was lost in animals (Zong et al., 2009).

The model plant *Arabidopsis thaliana* possesses six identifiable RDRs (Wassenegger and Krczal, 2006). The *RDR*α 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 *RDR*γ genes (*RDR3*, *RDR4*, and *RDR5*; also called *RDR3a*–*RDR3c*), 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 *RDR*γ gene in several other sequenced plant genomes, including rice (*Oryza sativa*), poplar (*Populus trichocarpa*), moss (*Physcomitrella*), and a lycophyte (*Selaginella*), as well as in 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

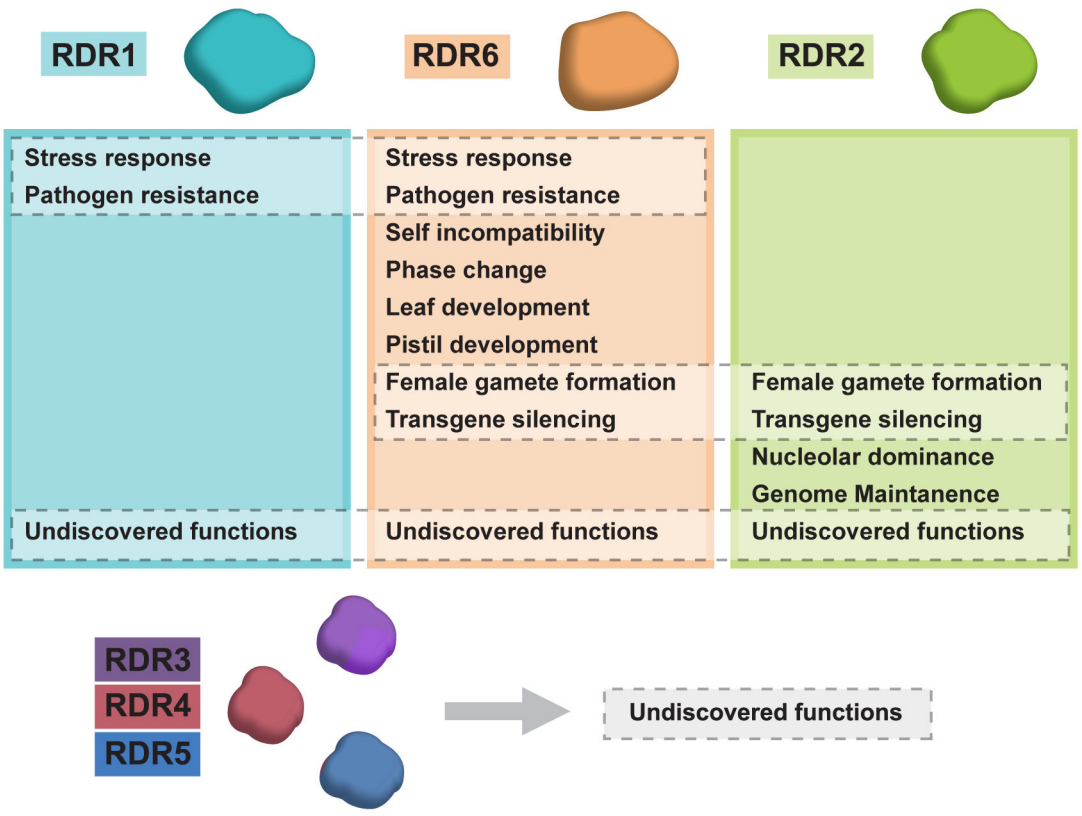

Figure 1. The functions of Arabidopsis RDRs.

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.

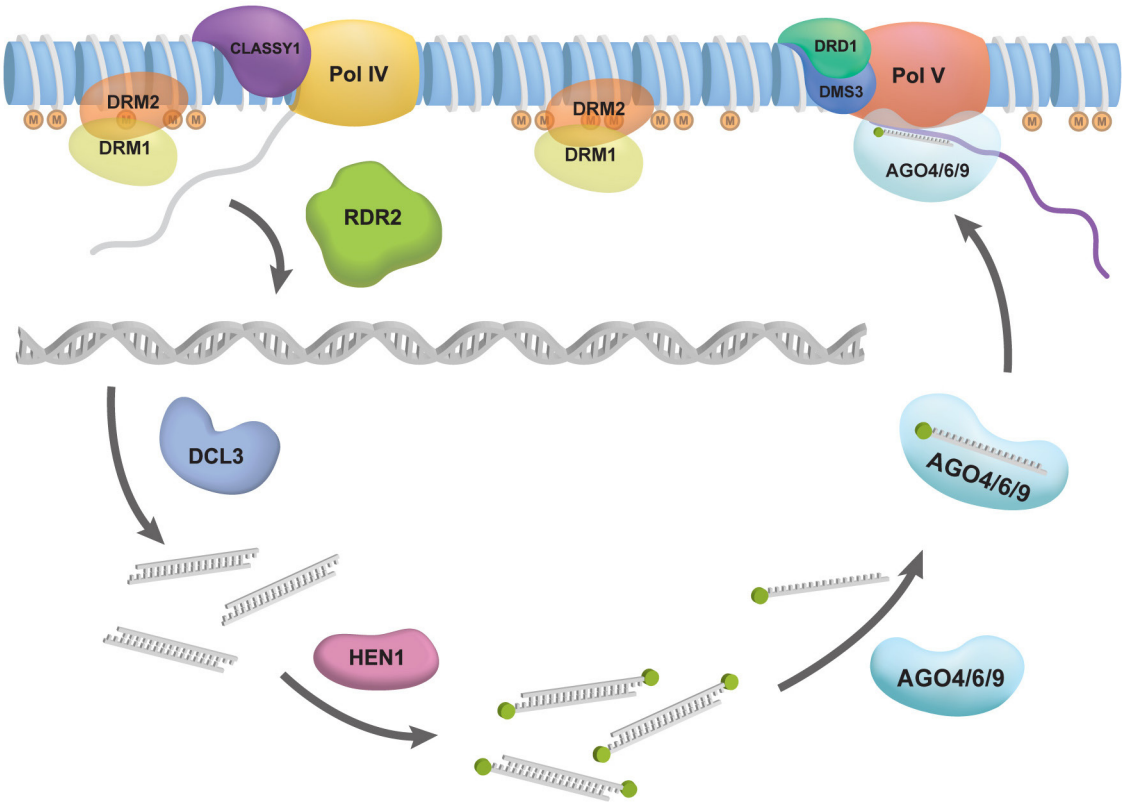

Figure 2. The RNA-directed DNA methylation pathway of Arabidopsis.

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 to 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).

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). RDR2-dependent 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 RDR2-dependent dsRNAs are processed by DCL3 into 24 nt hsiRNAs (Xie et al., 2004; Lu et al., 2006). These mostly 24 nt hsiRNAs are bound by an AGO4-, AGO6-, or AGO9-RISC, and target the silencing of complementary genomic loci, including the original locus (Zheng et al., 2007; Havecker et al., 2010; Olmedo-Monfil et al., 2010). Because heterochromatic regions are highly repetitive, many hsiRNAs are both cis- and trans-acting regulatory RNAs. Interestingly, recent evidence suggests that AGO9-RISC may incorporate RDR2-dependent hsiRNAs that specifically silence repetitive loci in developing female gametophytes (Olmedo-Monfil et al., 2010). We will discuss this RDR2 function in further detail below.

RdDM by hsiRNAs loaded in AGO4-, AGO6-, or AGO9-RISC is believed to also require non-coding RNAs that are generated by a second plant-specific DNA-dependent RNA polymerase (Pol

V) 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 AGO4/6/9-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 AGO4/6/9-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 (lsiRNAs) are dependent on DCL3 and Pol IV for their biogenesis, and can associate with AGO4 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 lsiRNA-directed DNA methylation and miRNA-mediated target RNA cleavage/degradation is required to produce complete post-transcriptional 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 AGO4-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 AGO1-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; Gasciolli 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 AGO7-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 AGO7-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 AGO1-RISC, go on to target *ARF3* and *4* transcripts for post-transcriptional 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 AGO1-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 AGO1-22 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 (Katiyar-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; Katiyar-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

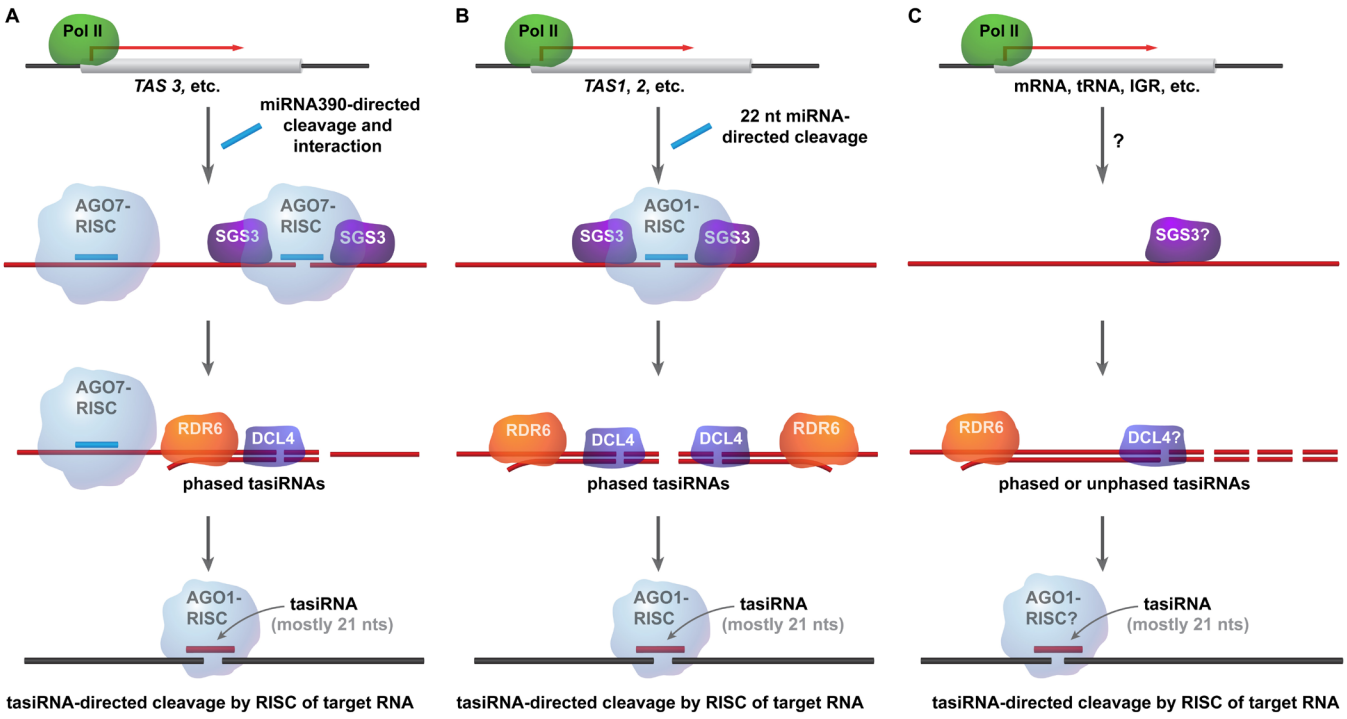

Figure 3. Models of RDR6-dependent ta-siRNA biogenesis.

(A) For *TAS3*, ta-siRNAs are processed from segments of this Pol II-produced non-coding RNA that are defined by two miR390 target sites, one that is cleavable (3' end of transcript) and the other that is not (5' end of RNA). These two target sites interact specifically with AGO7-RISC that is bound to a mature miR390. All current evidence suggests that the dual miRNA390 target sites, as well as the involvement of AGO7-RISC, results in the recognition of *TAS3* by RDR6, SGS3, and/or some other ta-siRNA biogenesis component to initiate phased, DCL4-dependent, 21 nt smRNA production from the cleaved end (3'). Once incorporated into an ARGONAUTE (AGO)-containing RISC, ta-siRNAs post-transcriptionally down-regulate protein-coding transcripts from unrelated loci in a fashion reminiscent of the miRNA-directed RNA silencing pathway.
(B) At *TAS1*, *2*, and *4*, ta-siRNAs are processed from segments of these Pol II-produced non-coding RNAs that are defined by 22nt miRNA-mediated AGO1-RISC cleavage. These cleaved substrates are converted to dsRNA by RDR6 with the help of its interacting partner SGS3. This dsRNA is successively processed by DCL4 in 21 nt increments from the initially cleaved end, resulting in the production of phased siRNAs. Once incorporated into an AGO-containing RISC, ta-siRNAs post-transcriptionally down-regulate protein-coding transcripts from unrelated loci in a fashion reminiscent of the miRNA-directed RNA silencing pathway.
(C) RDR6 is also required to produce phased or unphased siRNAs from RNAs that do not appear to be targeted by either mi- or siRNAs. These molecules may be recognized as smRNA-producing loci by the RDR6-interacting partner SGS3. RDR6-dependent production of dsRNA molecules at these loci results in the production of mostly 21 nt smRNAs that are likely processed by DCL4. These smRNAs are then possibly incorporated into an AGO-containing RISC to direct down-regulation of protein-coding transcripts from unrelated loci. It is worth noting that the mechanism behind RDR6 identification of these substrates is entirely unknown.

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

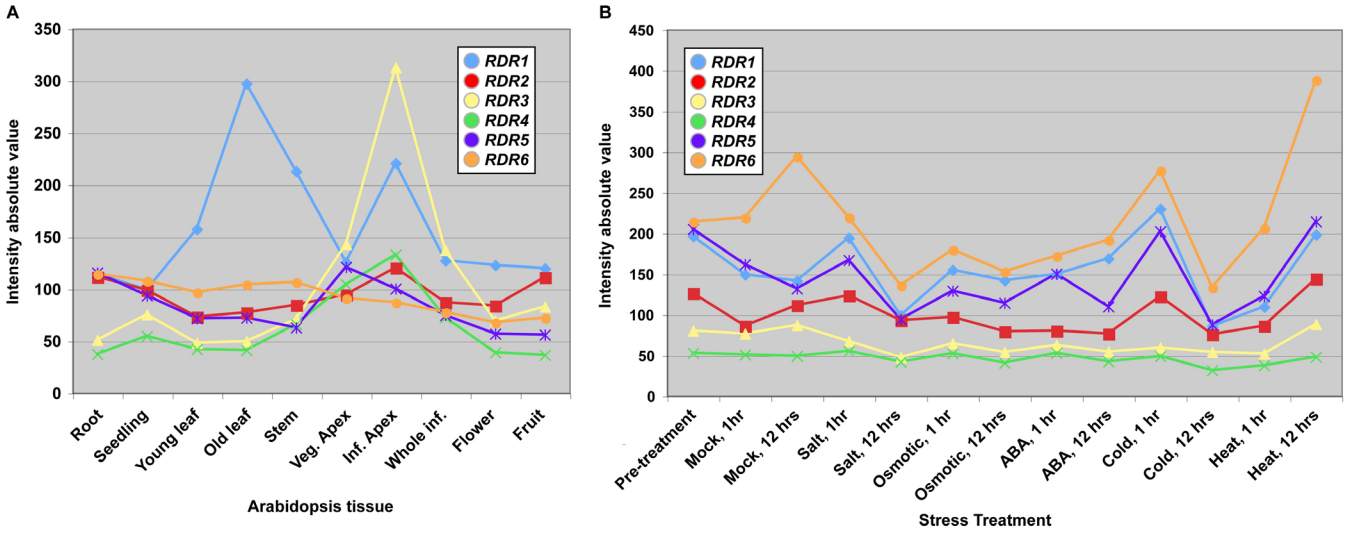

Figure 4. The expression patterns of all six Arabidopsis *RDR* genes in numerous tissues and stress conditions from tiling microarray experiments.

(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 rounder 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*.

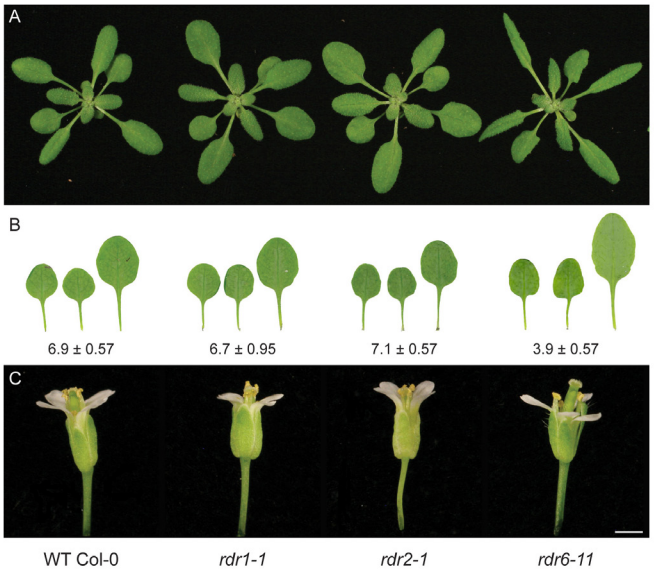

Figure 5. Some developmental phenotypes of *rdr6*.

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.

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

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). Because of 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; Yoshikawa 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 (Stewart et al., 2001; Scheible et al., 2004; Misson 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; Misson 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 exsertion, 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 megaspore 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 distinct megaspore 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 megaspore 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 megaspore 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 transcriptional 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 megaspore 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 megaspore mother cell, megaspores, 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 transcriptional 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; Schopfer 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 exsertion.

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 exsertion, 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 exsertion (Figure 5C), though this phenotype is further exaggerated in the *AlSRKb-AlSCRb* background. The exaggerated stigma exsertion 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-ARF2/3/4* pathway in *rdr6* is responsible for the self-incompatibility and stigma exsertion 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 exsertion 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 *KOKOPELLI* (*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 *ARIADNE14* (*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 (lbl1) and ragged seedling2 (rgd2), respectively — and RDR6 (shootless2, shl2) 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 *lbl1* 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 *MIR166* 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; Ratcliff 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; Muangsan 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; Muangsan 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; Mlotshwa 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.,

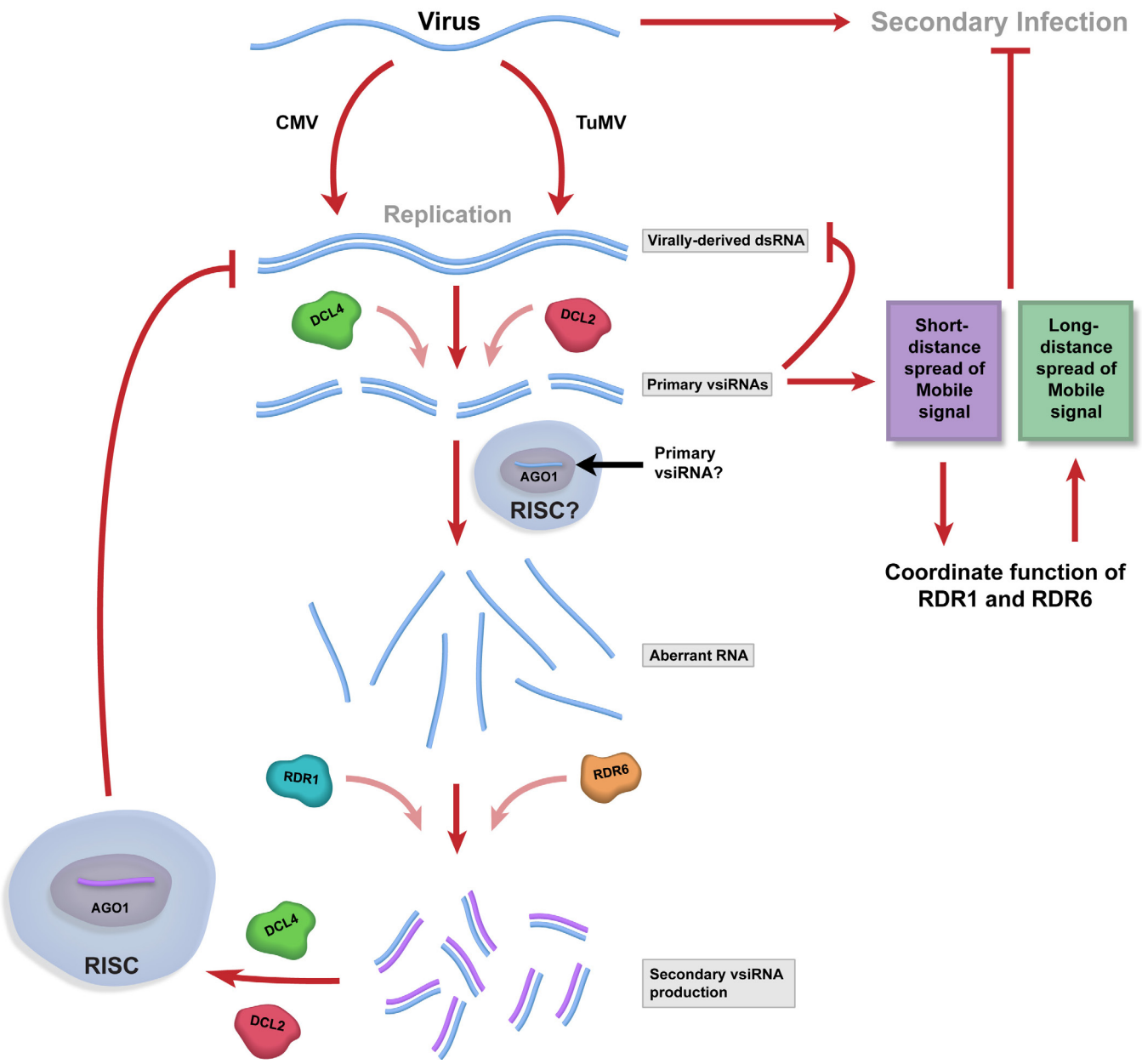

Figure 6. A model of RDR function in Arabidopsis anti-viral defense.

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 *TuMV*Δ*HC*). 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, *TuMV*Δ*HC* 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 (*CMV*Δ*2B*) 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 *CMV*Δ*2B*, 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; Mlotshwa 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 *TuMV*Δ*HC* 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 (Katiyar-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 (Katiyar-Agarwal et al., 2006; Katiyar-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* (Katiyar-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 Δ^1-pyroline-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 *TAS1* 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

Adenot, X., Elmayan, T., Lauressergues, D., Boutet, S., Bouche, N., Gasciolli, V., and Vaucheret, H. (2006). DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. Curr Biol **16:** 927-932.

Akbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., Zhang, P., Gruissem, W., Meins, F., Jr., Hohn, T., and Pooggin, M.M. (2006). Molecular characterization of geminivirus-derived small RNAs in different plant species. Nucleic Acids Res **34:** 462-471.

Al-Kaff, N.S., Covey, S.N., Kreike, M.M., Page, A.M., Pinder, R., and Dale, P.J. (1998). Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. Science **279:** 2113-2115.

Alamillo, J.M., Saenz, P., and Garcia, J.A. (2006). Salicylic acid-mediated and RNA-silencing defense mechanisms cooperate in the restriction of systemic spread of plum pox virus in tobacco. Plant J **48:** 217-227.

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121:** 207-221.

Almeida, R., and Allshire, R.C. (2005). RNA silencing and genome regulation. Trends Cell Biol **15:** 251-258.

Alvarado, V., and Scholthof, H.B. (2009). Plant responses against invasive nucleic acids: RNA silencing and its suppression by plant viral pathogens. Semin Cell Dev Biol **20:** 1032-1040.

Arazi, T., Talmor-Neiman, M., Stav, R., Riese, M., Huijser, P., and Baulcombe, D.C. (2005). Cloning and characterization of micro-RNAs from moss. Plant J **43:** 837-848.

Autran, D., Baroux, C., Raissig, M.T., Lenormand, T., Wittig, M., Grob, S., Steimer, A., Barann, M., Klostermeier, U.C., Leblanc, O., Vielle-Calzada, J.P., Rosenstiel, P., Grimanelli, D., and Grossniklaus, U. (2011). Maternal epigenetic pathways control parental contributions to Arabidopsis early embryogenesis. Cell **145:** 707-719.

Axtell, M.J., Snyder, J.A., and Bartel, D.P. (2007). Common functions for diverse small RNAs of land plants. Plant Cell **19:** 1750-1769.

Axtell, M.J., Jan, C., Rajagopalan, R., and Bartel, D.P. (2006). A two-hit trigger for siRNA biogenesis in plants. Cell **127:** 565-577.

Barrett, S.C. (2010). Understanding plant reproductive diversity. Philos Trans R Soc Lond B Biol Sci **365:** 99-109.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116:** 281-297.

Baulcombe, D. (2004). RNA silencing in plants. Nature **431:** 356-363.

Baulcombe, D.C., and English, J.J. (1996). Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. Curr Opin Biotech **7:** 173-180.

Baumberger, N., Tsai, C.H., Lie, M., Havecker, E., and Baulcombe, D.C. (2007). The Polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. Curr Biol **17:** 1609-1614.

Bi, Y.M., Wang, R.L., Zhu, T., and Rothstein, S.J. (2007). Global transcription profiling reveals differential responses to chronic nitrogen

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

Blevins, T., Rajeswaran, R., Shivaprasad, P.V., Beknazariants, D., Si-Ammour, A., Park, H.S., Vazquez, F., Robertson, D., Meins, F., Jr., Hohn, T., and Pooggin, M.M. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Res **34:** 6233-6246.

Boggs, N.A., Nasrallah, J.B., and Nasrallah, M.E. (2009a). Independent S-locus mutations caused self-fertility in *Arabidopsis thaliana*. PLoS Genet **5:** e1000426.

Boggs, N.A., Dwyer, K.G., Shah, P., McCulloch, A.A., Bechsgaard, J., Schierup, M.H., Nasrallah, M.E., and Nasrallah, J.B. (2009b). Expression of distinct self-incompatibility specificities in *Arabidopsis thaliana*. Genetics **182:** 1313-1321.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12:** 2383-2394.

Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., and Zhu, J.K. (2005). Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell **123:** 1279-1291.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., and Ziegler-Graff, V. (2007). The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Curr Biol **17:** 1615-1621.

Bouche, N., Lauressergues, D., Gasciolli, V., and Vaucheret, H. (2006). An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. Embo J **25:** 3347-3356.

Boutet, S., Vazquez, F., Liu, J., Beclin, C., Fagard, M., Gratias, A., Morel, J.B., Crete, P., Chen, X., and Vaucheret, H. (2003). Arabidopsis HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr Biol **13:** 843-848.

Buck, K.W. (1999). Replication of tobacco mosaic virus RNA. Philos Trans R Soc Lond B Biol Sci **354:** 613-627.

Burgyan, J., and Havelda, Z. (2011). Viral suppressors of RNA silencing. Trends Plant Sci **16:** 265-272.

Cardon, G., Hohmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterisation of the Arabidopsis SBP-box genes. Gene **237:** 91-104.

Cardon, G.H., Hohmann, S., Nettesheim, K., Saedler, H., and Huijser, P. (1997). Functional analysis of the *Arabidopsis thaliana* SBP-box gene SPL3: a novel gene involved in the floral transition. Plant J **12:** 367-377.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell **136:** 642-655.

Chan, S.W., Henderson, I.R., and Jacobsen, S.E. (2005). Gardening the genome: DNA methylation in *Arabidopsis thaliana*. Nat Rev Genet **6:** 351-360.

Chellappan, P., Xia, J., Zhou, X., Gao, S., Zhang, X., Coutino, G., Vazquez, F., Zhang, W., and Jin, H. (2010). siRNAs from miRNA sites mediate DNA methylation of target genes. Nucleic Acids Res **38:** 6883-6894.

Chen, H.M., Chen, L.T., Patel, K., Li, Y.H., Baulcombe, D.C., and Wu, S.H. (2010). 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants. Proc Natl Acad Sci USA **107:** 15269-15274.

Chien, J.C., and Sussex, I.M. (1996). Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by Gibberellins and photoperiod in *Arabidopsis thaliana* (L) Heynh. Plant Physiol **111:** 1321-1328.

Chitwood, D.H., Guo, M., Nogueira, F.T., and Timmermans, M.C. (2007). Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex. Development **134:** 813-823.

Chitwood, D.H., Nogueira, F.T., Howell, M.D., Montgomery, T.A., Carrington, J.C., and Timmermans, M.C. (2009). Pattern formation via small RNA mobility. Genes Dev **23:** 549-554.

Cuperus, J.T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R.T., Takeda, A., Sullivan, C.M., Gilbert, S.D., Montgomery, T.A., and Carrington, J.C. (2010). Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis. Nat Struct Mol Biol **17:** 997-1003.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101:** 543-553.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inze, D., Bennett, M.J., and Beeckman, T. (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development **134:** 681-690.

Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K.D., Carrington, J.C., and Voinnet, O. (2006). Hierarchical Action and Inhibition of Plant Dicer-Like Proteins in Antiviral Defense. Science **313:** 68-71.

Diaz-Pendon, J.A., and Ding, S.W. (2008). Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annu Rev Phytopathol **46:** 303-326.

Diaz-Pendon, J.A., Li, F., Li, W.X., and Ding, S.W. (2007). Suppression of antiviral silencing by cucumber mosaic virus 2b protein in Arabidopsis is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. Plant Cell **19:** 2053-2063.

Ding, S.W., and Voinnet, O. (2007). Antiviral immunity directed by small RNAs. Cell **130:** 413-426.

Donaire, L., Barajas, D., Martinez-Garcia, B., Martinez-Priego, L., Pagan, I., and Llave, C. (2008). Structural and genetic requirements for the biogenesis of tobacco rattle virus-derived small interfering RNAs. J Virol **82:** 5167-5177.

Donaire, L., Wang, Y., Gonzalez-Ibeas, D., Mayer, K.F., Aranda, M.A., and Llave, C. (2009). Deep-sequencing of plant viral small RNAs reveals effective and widespread targeting of viral genomes. Virology **392:** 203-214.

Dorweiler, J.E., Carey, C.C., Kubo, K.M., Hollick, J.B., Kermicle, J.L., and Chandler, V.L. (2000). mediator of paramutation1 is required for establishment and maintenance of paramutation at multiple maize loci. Plant Cell **12:** 2101-2118.

Douglas, R.N., Wiley, D., Sarkar, A., Springer, N., Timmermans, M.C., and Scanlon, M.J. (2010). ragged seedling2 Encodes an ARGONAUTE7-like protein required for mediolateral expansion, but not dorsiventrality, of maize leaves. Plant Cell **22:** 1441-1451.

Drews, G.N., and Yadegari, R. (2002). Development and function of the angiosperm female gametophyte. Annu Rev Genet **36:** 99-124.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova, S., Celenza, J., and Benkova, E. (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc Natl Acad Sci USA **105:** 8790-8794.

Dunoyer, P., Himber, C., and Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. Nat Genet **37:** 1356-1360.

Elmayan, T., Balzergue, S., Beon, F., Bourdon, V., Daubremet, J., Guenet, Y., Mourrain, P., Palauqui, J.C., Vernhettes, S., Vialle, T., Wostrikoff, K., and Vaucheret, H. (1998). Arabidopsis mutants impaired in cosuppression. Plant Cell **10:** 1747-1758.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., and Bowman, J.L. (2003). Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr Biol **13:** 1768-1774.

Eshed, Y., Baum, S.F., Perea, J.V., and Bowman, J.L. (2001). Establishment of polarity in lateral organs of plants. Curr Biol **11:** 1251-1260.

Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K., and Bowman, J.L. (2004). Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development **131:** 2997-3006.

Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., and Carrington, J.C. (2006). Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. Curr Biol **16:** 939-944.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic Acid for the induction of systemic acquired resistance. Science **261:** 754-756.

Garcia, D., Collier, S.A., Byrne, M.E., and Martienssen, R.A. (2006). Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway. Curr Biol **16:** 933-938.

Garcia-Ruiz, H., Takeda, A., Chapman, E.J., Sullivan, C.M., Fahlgren, N., Brempelis, K.J., and Carrington, J.C. (2010). Arabidopsis RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during Turnip Mosaic Virus infection. Plant Cell **22:** 481-496.

Gasciolli, V., Mallory, A.C., Bartel, D.P., and Vaucheret, H. (2005). Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. Curr Biol **15:** 1494-1500.

Gonzalez, A., Zhao, M., Leavitt, J.M., and Lloyd, A.M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant J **53:** 814-827.

Harvey, J.J., Lewsey, M.G., Patel, K., Westwood, J., Heimstadt, S., Carr, J.P., and Baulcombe, D.C. (2011). An antiviral defense role of AGO2 in plants. PLoS One **6:** e14639.

Havecker, E.R., Wallbridge, L.M., Hardcastle, T.J., Bush, M.S., Kelly, K.A., Dunn, R.M., Schwach, F., Doonan, J.H., and Baulcombe, D.C. (2010). The Arabidopsis RNA-directed DNA methylation argonautes functionally diverge based on their expression and interaction with target loci. Plant Cell **22:** 321-334.

He, J., Dong, Z., Jia, Z., Wang, J., and Wang, G. (2010). Isolation, expression and functional analysis of a putative RNA-dependent RNA polymerase gene from maize (Zea mays L.). Mol Biol Rep **37:** 865-874.

Henderson, D.C., Muehlbauer, G.J., and Scanlon, M.J. (2005). Radial leaves of the maize mutant ragged seedling2 retain dorsiventral anatomy. Dev Biol **282:** 455-466.

Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. (2005). RNA polymerase IV directs silencing of endogenous DNA. Science **308:** 118-120.

Hewezi, T., Howe, P., Maier, T.R., and Baum, T.J. (2008). Arabidopsis small RNAs and their targets during cyst nematode parasitism. Molecular Plant-Microbe Interactions **21:** 1622-1634.

Ho, T., Pallett, D., Rusholme, R., Dalmay, T., and Wang, H. (2006). A simplified method for cloning of short interfering RNAs from Brassica juncea infected with Turnip mosaic potyvirus and Turnip crinkle carmovirus. J Virol Methods **136:** 217-223.

Hsieh, L.C., Lin, S.I., Shih, A.C., Chen, J.W., Lin, W.Y., Tseng, C.Y., Li, W.H., and Chiou, T.J. (2009). Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol **151:** 2120-2132.

Hunter, C., Sun, H., and Poethig, R.S. (2003). The Arabidopsis heterochronic gene ZIPPY is an ARGONAUTE family member. Curr Biol **13:** 1734-1739.

Hunter, C., Willmann, M.R., Wu, G., Yoshikawa, M., de la Luz Gutierrez-Nava, M., and Poethig, S.R. (2006). Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in Arabidopsis. Development **133:** 2973-2981.

Jen, C.H., Michalopoulos, I., Westhead, D.R., and Meyer, P. (2005). Natural antisense transcripts with coding capacity in Arabidopsis may have a regulatory role that is not linked to double-stranded RNA degradation. Genome Biol **6:** R51.

Jia, Y., Lisch, D.R., Ohtsu, K., Scanlon, M.J., Nettleton, D., and Schnable, P.S. (2009). Loss of RNA-dependent RNA polymerase 2 (RDR2) function causes widespread and unexpected changes in the expression of transposons, genes, and 24-nt small RNAs. PLoS Genet **5:** e1000737.

Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAS and their regulatory roles in plants. Annu Rev Plant Biol **57:** 19-53.

Kachroo, A., Nasrallah, M.E., and Nasrallah, J.B. (2002). Self-incompatibility in the Brassicaceae: receptor-ligand signaling and cell-to-cell communication. Plant Cell **14 Suppl:** S227-238.

Kanno, T., Mette, M.F., Kreil, D.P., Aufsatz, W., Matzke, M., and Matzke, A.J. (2004). Involvement of putative SNF2 chromatin remodeling protein DRD1 in RNA-directed DNA methylation. Curr Biol **14:** 801-805.

Kanno, T., Huettel, B., Mette, M.F., Aufsatz, W., Jaligot, E., Daxinger, L., Kreil, D.P., Matzke, M., and Matzke, A.J. (2005). Atypical RNA polymerase subunits required for RNA-directed DNA methylation. Nat Genet **37:** 761-765.

Kanno, T., Bucher, E., Daxinger, L., Huettel, B., Bohmdorfer, G., Gregor, W., Kreil, D.P., Matzke, M., and Matzke, A.J. (2008). A structural-maintenance-of-chromosomes hinge domain-containing protein is required for RNA-directed DNA methylation. Nat Genet **40:** 670-675.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A., and Carrington, J.C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA unction. Dev Cell **4:** 205-217.

Kasschau, K.D., Fahlgren, N., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., and Carrington, J.C. (2007). Genome-wide profiling and analysis of Arabidopsis siRNAs. PLoS Biol **5:** e57.

Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A., and Jin, H. (2007). A novel class of bacteria-induced small RNAs in Arabidopsis. Genes Dev **21:** 3123-3134.

Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Jr., Zhu, J.K., Staskawicz, B.J., and Jin, H. (2006). A pathogen-inducible endogenous siRNA in plant immunity. Proc Natl Acad Sci U S A **103:** 18002-18007.

Kerstetter, R.A., Bollman, K., Taylor, R.A., Bomblies, K., and Poethig, R.S. (2001). KANADI regulates organ polarity in Arabidopsis. Nature **411:** 706-709.

Kidner, C.A., and Martienssen, R.A. (2004). Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature **428:** 81-84.

Koch, M.A., Haubold, B., and Mitchell-Olds, T. (2000). Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in Arabidopsis, Arabis, and related genera (Brassicaceae). Mol Biol Evol **17:** 1483-1498.

Kume, K., Tsutsumi, K., and Saitoh, Y. (2010). TAS1 trans-acting siRNA targets are differentially regulated at low temperature, and TAS1 trans-acting siRNA mediates temperature-controlled At1g51670 expression. Biosci Biotechnol Biochem **74:** 1435-1440.

Laskowski, M.J., Williams, M.E., Nusbaum, H.C., and Sussex, I.M. (1995). Formation of lateral root meristems is a two-stage process. De-

velopment **121:** 3303-3310.

Laubinger, S., Zeller, G., Henz, S.R., Sachsenberg, T., Widmer, C.K., Naouar, N., Vuylsteke, M., Scholkopf, B., Ratsch, G., and Weigel, D. (2008). At-TAX: a whole genome tiling array resource for developmental expression analysis and transcript identification in *Arabidopsis thaliana*. Genome Biol **9:** R112.

Law, J.A., and Jacobsen, S.E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet **11:** 204-220.

Lea, U.S., Slimestad, R., Smedvig, P., and Lillo, C. (2007). Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. Planta **225:** 1245-1253.

Li, A., Schuermann, D., Gallego, F., Kovalchuk, I., and Tinland, B. (2002). Repair of damaged DNA by Arabidopsis cell extract. Plant Cell **14:** 263-273.

Li, H., Xu, L., Wang, H., Yuan, Z., Cao, X., Yang, Z., Zhang, D., Xu, Y., and Huang, H. (2005). The Putative RNA-dependent RNA polymerase RDR6 acts synergistically with ASYMMETRIC LEAVES1 and 2 to repress BREVIPEDICELLUS and MicroRNA165/166 in Arabidopsis leaf development. Plant Cell **17:** 2157-2171.

Lillo, C., Lea, U.S., and Ruoff, P. (2008). Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. Plant Cell Environ **31:** 587-601.

Liu, B., Chen, Z., Song, X., Liu, C., Cui, X., Zhao, X., Fang, J., Xu, W., Zhang, H., Wang, X., Chu, C., Deng, X., Xue, Y., and Cao, X. (2007). Oryza sativa dicer-like4 reveals a key role for small interfering RNA silencing in plant development. Plant Cell **19:** 2705-2718.

Lu, C., Kulkarni, K., Souret, F.F., MuthuValliappan, R., Tej, S.S., Poethig, R.S., Henderson, I.R., Jacobsen, S.E., Wang, W., Green, P.J., and Meyers, B.C. (2006). MicroRNAs and other small RNAs enriched in the Arabidopsis RNA-dependent RNA polymerase-2 mutant. Genome Res **16:** 1276-1288.

Madsen, C.T., Stephens, J., Hornyik, C., Shaw, J., Collinge, D.B., Lacomme, C., and Albrechtsen, M. (2009). Identification and characterization of barley RNA-directed RNA polymerases. Biochim Biophys Acta **1789:** 375-385.

Marin, E., Jouannet, V., Herz, A., Lokerse, A.S., Weijers, D., Vaucheret, H., Nussaume, L., Crespi, M.D., and Maizel, A. (2010). miR390, Arabidopsis TAS3 ta-siRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. Plant Cell **22:** 1104-1117.

Matzke, M.A., and Matzke, A.J. (1995). Homology-dependent gene silencing in transgenic plants: what does it really tell us? Trends Genet **11:** 1-3.

McConnell, J.R., and Barton, M.K. (1998). Leaf polarity and meristem formation in Arabidopsis. Development **125:** 2935-2942.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001). Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature **411:** 709-713.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature **431:** 343-349.

Misson, J., Raghothama, K.G., Jain, A., Jouhet, J., Block, M.A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., and Thibaud, M.C. (2005). A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA **102:** 11934-11939.

Mlotshwa, S., Pruss, G.J., and Vance, V. (2008a). Small RNAs in viral infection and host defense. Trends Plant Sci **13:** 375-382.

Mlotshwa, S., Pruss, G.J., Peragine, A., Endres, M.W., Li, J., Chen, X., Poethig, R.S., Bowman, L.H., and Vance, V. (2008b). DICER-LIKE2 plays a primary role in transitive silencing of transgenes in Arabidopsis. PLoS ONE **3:** e1755.

Moissiard, G., Parizotto, E.A., Himber, C., and Voinnet, O. (2007). Transitivity in Arabidopsis can be primed, requires the redundant action of the antiviral Dicer-like 4 and Dicer-like 2, and is compromised by viral-encoded suppressor proteins. RNA **13:** 1268-1278.

Moldovan, D., Spriggs, A., Dennis, E.S., and Wilson, I.W. (2010). The hunt for hypoxia responsive natural antisense short interfering RNAs. Plant Signaling & Behavior **5:** 247.

Molnar, A., Csorba, T., Lakatos, L., Varallyay, E., Lacomme, C., and Burgyan, J. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J Virol **79:** 7812-7818.

Montgomery, T.A., Howell, M.D., Cuperus, J.T., Li, D., Hansen, J.E., Alexander, A.L., Chapman, E.J., Fahlgren, N., Allen, E., and Carrington, J.C. (2008a). Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell **133:** 128-141.

Montgomery, T.A., Yoo, S.J., Fahlgren, N., Gilbert, S.D., Howell, M.D., Sullivan, C.M., Alexander, A., Nguyen, G., Allen, E., Ahn, J.H., and Carrington, J.C. (2008b). AGO1-miR173 complex initiates phased siRNA formation in plants. Proc Natl Acad Sci USA **105:** 20055-20062.

Morcuende, R., Bari, R., Gibon, Y., Zheng, W., Pant, B.D., Blasing, O., Usadel, B., Czechowski, T., Udvardi, M.K., Stitt, M., and Scheible, W.R. (2007). Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. Plant Cell Environ **30:** 85-112.

Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell **14:** 629-639.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T.A., and Vaucheret, H. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:** 533-542.

Muangsan, N., Beclin, C., Vaucheret, H., and Robertson, D. (2004). Geminivirus VIGS of endogenous genes required SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. Plant J. **38,** 1004-1014.

Murphy, A.M., and Carr, J.P. (2002). Salicylic acid has cell-specific effects on tobacco mosaic virus replication and cell-to-cell movement. Plant Physiol **128:** 552-563.

Nagasaki, H., Itoh, J., Hayashi, K., Hibara, K., Satoh-Nagasawa, N., Nosaka, M., Mukouhata, M., Ashikari, M., Kitano, H., Matsuoka, M., Nagato, Y., and Sato, Y. (2007). The small interfering RNA production pathway is required for shoot meristem initiation in rice. Proc Natl Acad Sci USA **104:** 14867-14871.

Nasrallah, M.E., Liu, P., and Nasrallah, J.B. (2002). Generation of self-incompatible *Arabidopsis thaliana* by transfer of two S locus genes from A. lyrata. Science **297:** 247-249.

Nasrallah, M.E., Liu, P., Sherman-Broyles, S., Boggs, N.A., and Nasrallah, J.B. (2004). Natural variation in expression of self-incompatibility in *Arabidopsis thaliana:* implications for the evolution of selfing. Proc Natl Acad Sci USA **101:** 16070-16074.

Naylor, M., Murphy, A.M., Berry, J.O., and Carr, J.P. (1998). Salicylic acid can induce resistance to plant virus movement. Mol Plant Microbe Interact **11:** 860-868.

Nogueira, F.T., Madi, S., Chitwood, D.H., Juarez, M.T., and Timmermans, M.C. (2007). Two small regulatory RNAs establish opposing

fates of a developmental axis. Genes Dev **21:** 750-755.

Nogueira, F.T., Chitwood, D.H., Madi, S., Ohtsu, K., Schnable, P.S., Scanlon, M.J., and Timmermans, M.C. (2009). Regulation of small RNA accumulation in the maize shoot apex. PLoS Genet **5:** e1000320.

Olmedo-Monfil, V., Duran-Figueroa, N., Arteaga-Vazquez, M., Demesa-Arevalo, E., Autran, D., Grimanelli, D., Slotkin, R.K., Martienssen, R.A., and Vielle-Calzada, J.P. (2010). Control of female gamete formation by a small RNA pathway in Arabidopsis. Nature **464:** 628-632.

Olsen, K.M., Slimestad, R., Lea, U.S., Brede, C., Lovdal, T., Ruoff, P., Verheul, M., and Lillo, C. (2009). Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. Plant Cell Environ **32:** 286-299.

Onodera, Y., Haag, J.R., Ream, T., Nunes, P.C., Pontes, O., and Pikaard, C.S. (2005). Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell **120:** 613-622.

Pandey, S.P., and Baldwin, I.T. (2007). RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of Nicotiana attenuata to herbivore attack in nature. Plant J **50:** 40-53.

Pandey, S.P., Gaquerel, E., Gase, K., and Baldwin, I.T. (2008). RNA-directed RNA polymerase3 from Nicotiana attenuata is required for competitive growth in natural environments. Plant Physiol **147:** 1212-1224.

Pantaleo, V., Szittya, G., and Burgyan, J. (2007). Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. J Virol **81:** 3797-3806.

Pekker, I., Alvarez, J.P., and Eshed, Y. (2005). Auxin response factors mediate Arabidopsis organ asymmetry via modulation of KANADI activity. Plant Cell **17:** 2899-2910.

Peng, M., Bi, Y.M., Zhu, T., and Rothstein, S.J. (2007). Genome-wide analysis of Arabidopsis responsive transcriptome to nitrogen limitation and its regulation by the ubiquitin ligase gene NLA. Plant Mol Biol **65:** 775-797.

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H.L., and Poethig, R.S. (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. Genes Dev **18:** 2368-2379.

Poethig, R.S. (2003). Phase change and the regulation of developmental timing in plants. Science **301:** 334-336.

Poethig, R.S. (2010). The past, present, and future of vegetative phase change. Plant Physiol **154:** 541-544.

Pogue, G.P., Huntley, C.C., and Hall, T.C. (1994). Common replication strategies emerging from the study of diverse groups of positive-strand RNA viruses. Arch Virol Suppl **9:** 181-194.

Pontier, D., Yahubyan, G., Vega, D., Bulski, A., Saez-Vasquez, J., Hakimi, M.A., Lerbs-Mache, S., Colot, V., and Lagrange, T. (2005). Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in Arabidopsis. Genes Dev **19:** 2030-2040.

Pulido, A., and Laufs, P. (2010). Co-ordination of developmental processes by small RNAs during leaf development. J Exp Bot **61:** 1277-1291.

Qi, X., Bao, F.S., and Xie, Z. (2009). Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA-dependent RNA polymerases in viral siRNA biogenesis. PLoS One **4:** e4971.

Qi, Y., He, X., Wang, X.J., Kohany, O., Jurka, J., and Hannon, G.J. (2006). Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation. Nature **443:** 1008-1012.

Qu, F., Ye, X., and Morris, T.J. (2008). Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. Proc Natl Acad Sci USA **105:** 14732-14737.

Qu, F., Ye, X., Hou, G., Sato, S., Clemente, T.E., and Morris, T.J. (2005). RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in Nicotiana benthamiana. J Virol **79:** 15209-15217.

Rajagopalan, R., Vaucheret, H., Trejo, J., and Bartel, D.P. (2006). A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes Dev **20:** 3407-3425.

Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. (1997). A similarity between viral defense and gene silencing in plants. Science **276:** 1558-1560.

Ron, M., Alandete Saez, M., Eshed Williams, L., Fletcher, J.C., and McCormick, S. (2010). Proper regulation of a sperm-specific cis-nat-siRNA is essential for double fertilization in Arabidopsis. Genes Dev **24:** 1010-1021.

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.

Scheible, W.R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M.K., and Stitt, M. (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol **136:** 2483-2499.

Schopfer, C.R., Nasrallah, M.E., and Nasrallah, J.B. (1999). The male determinant of self-incompatibility in Brassica. Science **286:** 1697-1700.

Schwab, R., Maizel, A., Ruiz-Ferrer, V., Garcia, D., Bayer, M., Crespi, M., Voinnet, O., and Martienssen, R.A. (2009). Endogenous Ta-siRNAs mediate non-cell autonomous effects on gene regulation in *Arabidopsis thaliana*. PLoS One **4:** e5980.

Schwach, F., Vaistij, F.E., Jones, L., and Baulcombe, D.C. (2005). An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol **138:** 1842-1852.

Siegfried, K.R., Eshed, Y., Baum, S.F., Otsuga, D., Drews, G.N., and Bowman, J.L. (1999). Members of the YABBY gene family specify abaxial cell fate in Arabidopsis. Development **126:** 4117-4128.

Stein, J.C., Howlett, B., Boyes, D.C., Nasrallah, M.E., and Nasrallah, J.B. (1991). Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of Brassica oleracea. Proc Natl Acad Sci USA **88:** 8816-8820.

Stewart, A.J., Chapman, W., Jenkins, G.I., Graham, I., Martin, T., and Crozier, A. (2001). The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. Plant Cell and Environment **24:** 1189-1197.

Szakonyi, D., Moschopoulos, A., and Byrne, M.E. (2010). Perspectives on leaf dorsoventral polarity. J Plant Res **123:** 281-290.

Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A., and Hinata, K. (2000). The S receptor kinase determines self-incompatibility in Brassica stigma. Nature **403:** 913-916.

Takayama, S., Shimosato, H., Shiba, H., Funato, M., Che, F.S., Watanabe, M., Iwano, M., and Isogai, A. (2001). Direct ligand-receptor complex interaction controls Brassica self-incompatibility. Nature **413:** 534-538.

Takayama, S., Shiba, H., Iwano, M., Shimosato, H., Che, F.S., Kai, N., Watanabe, M., Suzuki, G., Hinata, K., and Isogai, A. (2000). The pollen determinant of self-incompatibility in Brassica campestris. Proc Natl Acad Sci USA **97:** 1920-1925.

Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M., and Watanabe, Y. (2008). The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. Plant Cell Physiol **49:**

493-500.

Talmor-Neiman, M., Stav, R., Klipcan, L., Buxdorf, K., Baulcombe, D.C., and Arazi, T. (2006). Identification of trans-acting siRNAs in moss and an RNA-dependent RNA polymerase required for their biogenesis. Plant J **48:** 511-521.

Tang, G., Reinhart, B.J., Bartel, D.P., and Zamore, P.D. (2003). A biochemical framework for RNA silencing in plants. Genes Dev **17:** 49-63.

Tantikanjana, T., Rizvi, N., Nasrallah, M.E., and Nasrallah, J.B. (2009). A dual role for the S-locus receptor kinase in self-incompatibility and pistil development revealed by an Arabidopsis rdr6 mutation. Plant Cell **21:** 2642-2654.

Telfer, A., Bollman, K.M., and Poethig, R.S. (1997). Phase change and the regulation of trichome distribution in *Arabidopsis thaliana.* Development **124:** 645-654.

Timmermans, M.C., Schultes, N.P., Jankovsky, J.P., and Nelson, T. (1998). Leafbladeless1 is required for dorsoventrality of lateral organs in maize. Development **125:** 2813-2823.

Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Noji, M., Yamazaki, M., and Saito, K. (2005). Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J **42:** 218-235.

Tomari, Y., and Zamore, P.D. (2005). Perspective: machines for RNAi. Genes Dev **19:** 517-529.

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A.C., Hilbert, J.L., Bartel, D.P., and Crete, P. (2004). Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol Cell **16:** 69-79.

Voinnet, O. (2005). Non-cell autonomous RNA silencing. FEBS Lett **579:** 5858-5871.

Voinnet, O. (2008). Use, tolerance and avoidance of amplified RNA silencing by plants. Trends Plant Sci **13:** 317-328.

Vrbsky, J., Akimcheva, S., Watson, J.M., Turner, T.L., Daxinger, L., Vyskot, B., Aufsatz, W., and Riha, K. (2010). siRNA-mediated methylation of Arabidopsis telomeres. PLoS Genet **6:** e1000986.

Wang, J., Gao, X., Li, L., Shi, X., Zhang, J., and Shi, Z. (2010a). Overexpression of Osta-siR2141 caused abnormal polarity establishment and retarded growth in rice. J Exp Bot **61:** 1885-1895.

Wang, J.W., Czech, B., and Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana.* Cell **138:** 738-749.

Wang, X.B., Wu, Q., Ito, T., Cillo, F., Li, W.X., Chen, X., Yu, J.L., and Ding, S.W. (2010b). RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana.* Proc Natl Acad Sci USA **107:** 484-489.

Wang, X.B., Jovel, J., Udomporn, P., Wang, Y., Wu, Q., Li, W.X., Gasciolli, V., Vaucheret, H., and Ding, S.W. (2011). The 21-Nucleotide, but Not 22-Nucleotide, Viral Secondary Small Interfering RNAs Direct Potent Antiviral Defense by Two Cooperative Argonautes in *Arabidopsis thaliana.* Plant Cell **23:** 1625-1638.

Wang, X.J., Gaasterland, T., and Chua, N.H. (2005). Genome-wide prediction and identification of cis-natural antisense transcripts in *Arabidopsis thaliana.* Genome Biol **6:** R30.

Wassenegger, M., and Krczal, G. (2006). Nomenclature and functions of RNA-directed RNA polymerases. Trends Plant Sci **11:** 142-151.

Wierzbicki, A.T., Haag, J.R., and Pikaard, C.S. (2008). Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. Cell **135:** 635-648.

Wierzbicki, A.T., Ream, T.S., Haag, J.R., and Pikaard, C.S. (2009). RNA polymerase V transcription guides ARGONAUTE4 to chromatin. Nat Genet **41:** 630-634.

Williams, L., Carles, C.C., Osmont, K.S., and Fletcher, J.C. (2005). A database analysis method identifies an endogenous trans-acting short-interfering RNA that targets the Arabidopsis ARF2, ARF3, and ARF4 genes. Proc Natl Acad Sci USA **102:** 9703-9708.

Willmann, M.R., and Poethig, R.S. (2011). The effect of the floral repressor FLC on the timing and progression of vegetative phase change in Arabidopsis. Development **138:** 677-685.

Wu, G., and Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. Development **133:** 3539-3547.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., and Poethig, R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138:** 750-759.

Wu, L., Zhou, H., Zhang, Q., Zhang, J., Ni, F., Liu, C., and Qi, Y. (2010). DNA methylation mediated by a microRNA pathway. Mol Cell **38:** 465-475.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. (2004). Genetic and functional diversification of small RNA pathways in plants. PLoS Biol **2:** E104.

Xu, L., Yang, L., Pi, L., Liu, Q., Ling, Q., Wang, H., Poethig, R.S., and Huang, H. (2006). Genetic interaction between the AS1-AS2 and RDR6-SGS3-AGO7 pathways for leaf morphogenesis. Plant Cell Physiol **47,** 853-863.

Yamaguchi, A., Wu, M.F., Yang, L., Wu, G., Poethig, R.S., and Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of LEAFY, FRUITFULL, and APETALA1. Dev Cell **17:** 268-278.

Yang, H., Wang, M., Gao, Z., Zhu, C., and Guo, X. (2010). Isolation of a novel RNA-dependent RNA polymerase 6 from Nicotiana glutinosa, NgRDR6, and analysis of its response to biotic and abiotic stresses. Mol Biol Rep **38:** 929-937.

Yang, J.H., Seo, H.H., Han, S.J., Yoon, E.K., Yang, M.S., and Lee, W.S. (2008). Phytohormone abscisic acid control RNA-dependent RNA polymerase 6 gene expression and post-transcriptional gene silencing in rice cells. Nucleic Acids Res **36:** 1220-1226.

Yang, S.J., Carter, S.A., Cole, A.B., Cheng, N.H., and Nelson, R.S. (2004). A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by Nicotiana benthamiana. Proc Natl Acad Sci USA **101:** 6297-6302.

Yao, X., Wang, H., Li, H., Yuan, Z., Li, F., Yang, L., and Huang, H. (2009). Two types of cis-acting elements control the abaxial epidermis-specific transcription of the MIR165a and MIR166a genes. FEBS Lett **583:** 3711-3717.

Yao, Y., Bilichak, A., Golubov, A., Blevins, T., and Kovalchuk, I. (2010). Differential sensitivity of Arabidopsis siRNA biogenesis mutants to genotoxic stress. Plant Cell Rep **29:** 1401-1410.

Yoon, E.K., Yang, J.H., Lim, J., Kim, S.H., Kim, S.K., and Lee, W.S. (2010). Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in Arabidopsis lateral root development. Nucleic Acids Res **38:** 1382-1391.

Yoshikawa, M., Peragine, A., Park, M.Y., and Poethig, R.S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. Genes Dev **19:** 2164-2175.

Yu, D., Fan, B., MacFarlane, S.A., and Chen, Z. (2003). Analysis of the involvement of an inducible Arabidopsis RNA-dependent RNA polymerase in antiviral defense. Mol Plant Microbe Interact **16:** 206-216.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J., and Chua, N.H. (2006). Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant de-

fense. Genes Dev **20:** 3255-3268.

Zheng, Q., Ryvkin, P., Li, F., Dragomir, I., Valladares, O., Yang, J., Cao, K., Wang, L.S., and Gregory, B.D. (2010). Genome-Wide Double-Stranded RNA Sequencing Reveals the Functional Significance of Base-Paired RNAs in Arabidopsis. PLoS Genet **6:** e1001141.

Zheng, X., Zhu, J., Kapoor, A., and Zhu, J.K. (2007). Role of Arabidopsis AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. EMBO J **26:** 1691-1701.

Zilberman, D. (2011). Balancing parental contributions in plant embryonic gene activation. Dev Cell **20:** 735-736.

Zong, J., Yao, X., Yin, J., Zhang, D., and Ma, H. (2009). Evolution of the RNA-dependent RNA polymerase (RdRP) genes: duplications and possible losses before and after the divergence of major eukaryotic groups. Gene **447:** 29-39.