THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

EFFECTS OF SEQUENCE COMPLEMENTARITY AND ARGONAUTE SLICING ACTIVITY ON RNA-DEPENDENT DNA METHYLATION IN ARABIDOPSIS

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ABSTRACT

RNA-directed DNA Methylation (RdDM) is an important regulator of gene expression. In plants, RdDM involves the Argonaute 4 (AGO4) family of proteins, as well as 24 nucleotide (nt) short RNAs. AGO4 is known to have a domain with slicing activity towards bound RNAs, but the significance of this slicing activity is not known. Likewise, previous work has shown that 24nt RNAs guide RdDM based on complementarity with the DNA to be methylated, but the degree of complementarity required is unknown. Here we examine several methods to further investigate these mechanisms. We show that an AGO4 transgene with three slicing catalytic residues altered (AGO4-AAA) does not accumulate sufficiently *in vivo* to test its efficacy. A 24nt RNA generating transgene based on rice 24nt microRNAs is also found not to accumulate sufficiently *in vivo* for further use as a system for testing the complementarity requirements of RdDM. A third system, based on inverted repeat RNA (irRNA) is shown to successfully accumulate and induce RdDM at a target locus, opening the irRNA system as a basis to test RdDM complementarity requirements in future experiments.

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Chapter 1

Introduction

Current Model of RNA Directed DNA Methylation

DNA methylation is an important regulator of gene expression, involved in both constitutive heterochromatin and the tissue-specific repression of genes (Chinnusamy and Zhu, 2011). In Arabidopsis, over one third of methylated loci appear to be controlled by short interfering RNA (siRNA) (Chinnusamy and Zhu, 2011). siRNA contribute to a process known as RNA directed DNA methylation (RdDM), which prominently involves some members of the Argonaute (AGO) family of proteins (Cokus et al., 2008; Chinnusamy and Zhu, 2011).

siRNA can be generated from endogenous or exogenous sources. The first observation of what was later found to be an siRNA-mediated phenotype was in petunias after artificial introduction of a chimeric chalcone synthase (CHS) gene (Napoli et al., 1990). In general, siRNA are commonly produced in response to the introduction of foreign RNA into an organism, whether from a viral or artificial source. Later experiments revealed the ubiquitous production of siRNAs from endogenous loci, commonly from heterochromatic regions. The siRNA derived from heterochromatic regions are known as heterochromatic siRNAs (hcRNAs), sometimes called cis-acting siRNAs (casiRNAs) or repeat-associated siRNAs (rasiRNAs) and are typically 24 nucleotides in length (Chinnusamy and Zhu, 2011).

Regardless of the source, the critical prerequisite for siRNA formation is the generation of double stranded RNA (dsRNA). In the case of viruses, dsRNA is commonly an intermediate in the replication of the viral genome. For endogenous siRNAs, dsRNA structure is sometimes

achieved via long antisense sequences in an RNA transcript, leading to the formation of a hairpin structure, with the tail of the hairpin forming effective dsRNA (Chinnusamy and Zhu, 2011).

In the case of most endogenous siRNAs, including hcRNAs, the formation of dsRNA requires an RNA-dependent RNA polymerase (RDR), RDR2 in the case of RdDM. In the biogenesis of hcRNA, the template for RDR2 is RNA transcribed from heterochromatic regions by Pol IV, a plant RNA polymerase that is poorly understood (Chinnusamy and Zhu, 2011). RDR2 is critical for RdDM; one study found that methylation of the SINE retroelement AtSN1 was significantly reduced at non-CG loci in the rdr2-1 mutant (Xie et al., 2004). The reason double-stranded RNA (and hence RDR) is required is to serve as a substrate for Dicer and Dicer-Like Proteins (DCL).

Once dsRNA has been formed, it is cleaved at intervals independent of its sequence by a dicer or DCL protein, DCL3 in the case of RdDM, to form short RNA duplexes. Each short RNA strand in these duplexes can then function as an hcRNA. One of these strands is loaded onto an AGO protein, which then mediates one of several suppression pathways. There are different AGO proteins for each pathway; in the case of hcRNA directed methylation, AGO4, AGO6, and AGO9, collectively known as AGO4 family proteins are involved (Chinnusamy and Zhu, 2011).

To produce a target for the hcRNA-AGO complex to interact with, plants utilize another RNA polymerase known as pol V. pol V shares the same second largest subunit, NRPE2, with pol IV (Ream et al., 2009). pol V produces ~200 nucleotide transcripts from noncoding heterochromatin and euchromatin. This process also requires the chromatin-remodeling complex DRD1 (Pontes et al., 2006).

The hcRNA-AGO complex can associate with the C-terminal domain of pol V and scan the nascent transcript for sequences matching the hcRNA. If the hcRNA recognizes a sequence in the transcript, the complex can recruit other proteins involved in methylation to the site. This initial recognition can recruit pol IV, RDR2, and DCL3, which can produce more hcRNAs which

in turn can direct methylation of downstream regions or of similar regions elsewhere in the genome (Chinnusamy and Zhu, 2011). A characteristic indicator of RdDM, in contrast to other methylation pathways, is the presence of asymmetric CHH (H stands for A, C, or T) methylation (Cao and Jacobsen, 2002). A summary of the current model of RdDM can be seen in Figure 1.

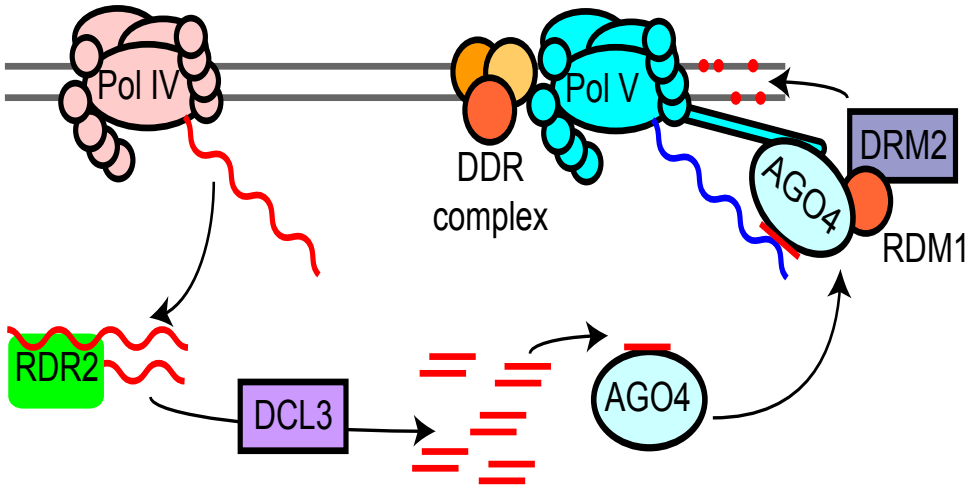

Figure 1: Summary of RdDM. The arrows follow Pol IV transcript synthesis, RDR2 dsRNA synthesis, and DCL processing followed by AGO4 loading, scanning of Pol V transcript, and direction of DNA methylation. Figure from Axtell (2013).

Importance of RdDM

One of the biggest roles of RdDM is the repression of transposons. Methylation has been shown to be more abundant in heterochromatic regions, with about 37% of methylated loci in heterochromatin shown to be associated with siRNA clusters (Zhang et al., 2006). When methylation was lost in mutants such as ddm1, there was widespread reactivation and transcription of transposons and pseudogenes (Miura et al., 2001)

Though transposons silencing is a major function of RdDM, it also functions in nucleolar dominance, gene regulation, stress response, and development (Chinnusamy and Zhu, 2011). For

instance, epialleles of *Flowering Locus C* (*FLC*) *were* found to be caused by RdDM. *Methylated Region near the Promoter of FLC* (*MPF*) led to the production of siRNAs, which in some cases directed methylation and heterochromatization of MPF and repression of FLC, leading to variation in flowering time (Zhai et al., 2008).

RdDM also has the potential for biotechnology applications, for instance in plant breeding and agriculture. RdDM of specific loci can be triggered by exogenous introduction of dsRNA matching the locus to be methylated, often via a viral vector. The resulting methylation represses target loci, is heritable, and does not require the introduction of a transgene into the plant. Thus, RdDM stands to become a powerful tool in the precise alteration of plant phenotypes for agricultural and other applications (Kasai and Kanazawa, 2013).

Importance of Current Research

Though research on RdDM has progressed rapidly in recent years, it is currently unclear how well the siRNA matches the transcript to induce this response. Furthermore, the AGO protein contains a slicing domain, which cleaves the siRNA-bound portion of the nascent transcript. This is thought to generate substrates for RDR2 and further generation of 24nt siRNAs, which could promote further RdDM. However, this has not yet been demonstrated (Chinnusamy and Zhu, 2011). Current experiments into RdDM involve the use of inverted repeat RNAs (irRNAs) . These are RNAs with sequences that are the reverse complement of each other, so that they form stretches of dsRNA, which can initiate the process of RdDM of the DNA sequence corresponding to the RNA. Thus, transgenes containing irRNAs can be used to study the RdDM of corresponding genes (Zilberman et al, 2004)

An alternative method of inducing DNA methylation was found in rice (Wu et al., 2010). A group of 24 nucleotide rice microRNAs (24nt miRNAs) was found to be cleaved by DCL3 and

loaded into AGO4 to direct DNA methylation, similar to the usual siRNAs in RdDM. This system has not yet been investigated for engineering, but if it worked it could be used to induce RdDM with precise sequences, rather than randomly cleaved 24nt RNAs derived from irRNAs.

This thesis describes experiments designed to elucidate the mechanism of RNA-directed DNA methylation, specifically the base-pairing requirements of the 24nt siRNA with the target site and the effect of AGO4 slicing on the accumulation of small RNAs. The overarching hypothesis is that most but not all of the nucleotides in the 24nt siRNA must be complementary for efficient methylation, and that AGO4 slicing activity is necessary for high accumulation of 24nt siRNA transcripts. For the work presented, the hypothesis is that AGO4 slicing activity is necessary for normal RdDM phenotypes, that rice-derived 24nt miRNAs can be engineered to induce RdDM at a target locus, and that an irRNA transgene can likewise be engineered to induce RdDM at a target locus. This work will help increase our understanding of an important regulator of gene expression in eukaryotes, enabling future work and biotechnology.

Chapter 2

Methods

To test the effect of removing slicing activity from AGO4, a critical Asp-Asp-His (DDH) motif was changed to a nonfunctional Ala-Ala-Ala (AAA) sequence. A resulting pAGO4::FLAG-AGO4-AAA vector was produced, with an AGO4 promoter driving a FLAG tagged, slicing deficient AGO4 protein. An analogous pAGO4::FLAG-wtAGO4 vector encoding the wild type, slicing functional AGO4 protein and an empty vector not encoding a transgene were also produced as controls.

These vectors were transformed into both *ago4-4* and *ago4-4/6-2/9-1* mutants to eliminate endogenous AGO4 activity. The triple mutant was used because AGO6 and AGO9 are both in the AGO4 family of proteins, so the triple mutant background ensures no AGO4 or AGO4-like activity except for the transgenic protein. The accumulation of the transgenic AGO4s was then assessed using a western blot with an anti-FLAG antibody.

A preliminary assessment of the effects of the slicing ability of AGO4 was then performed via bisulfite sequencing of pooled genomic samples at the *MEA-ISR* and *AtSN1* loci. The loci were chosen because of previous research characterizing their methylation profiles (Qi et al., 2006; Havecker et al., 2010). Bisulfite conversion of the DNA was performed using the Zymo EZ DNA Methylation kit, and Sanger Sequencing of the converted DNA conducted by the Huck Institute of Life Sciences Genomic Core Facility. MacVector software was then used to compare the sequence of the bisulfite converted DNA with the sequence of the unconverted loci, in order to infer methylation profiles. CG methylation, which is not RdDM dependent, was then compared to CHH and CHG methylation, which involve RdDM.

To test the RNA sequence complementarity requirements for RdDM, the 24nt rice miRNA genes discovered by Wu et al. were explored as a possible basis for transgenic miRNAs with varying degrees of complementarity to a methylation target. First, three of the 24nt miRNAs, *Osa-MIR1862*, *Osa-MIR1863* and *Osa-MIR1876*, were infiltrated into *Nicotiana benthamiana* and transformed into *Arabidopsis thaliana* to assay their accumulation. Each miRNA gene was driven by the 35S promoter. The accumulation of various sizes of small RNAs was then assessed with a northern blot.

MIR1682, which had the least accumulation of non-24nt RNA (which would confound the experiment by also interfering with the target site), was then used as the basis for two artificial miRNAs. The artificial miRNAs were designed to target two tandem repeats, SINE1 and SINE2, which are present at the relevant *FWA* locus in late-flowering *fwa-d* epimutants (Soppe et al., 2000). The miRNA sequence in the *MIR1682* gene was replaced 24nt sequences (and their inverse) matching the SINE1 and SINE2 elements so that the miRNA produced would target these elements at the *FWA* locus. Both artificial miRNA transgenes and the original *MIR1682* were then transformed into *fwa-d* plants, and the flowering times of T1 plants recorded as a proxy for remethylation at the *FWA* locus. None of the T1 transformant groups had significantly shorter flowering times, so the expression of 24nt miRNA in the earliest flowering transformants was assessed using a northern blot to determine if the miRNA was ineffective or simply not expressed.

As an alternative method of inducing RdDM, inverted repeat RNAs (irRNAs) have been previously studied in *fwa-d* plants (Kinoshita et al., 2006). To confirm the feasibility of using irRNAs to induce RdDM and repress the late-flowering *fwa-d* phenotype, a transgene driven by 35S promoters and with *FWA*-inverted repeat sequences was transformed into *fwa-d* plants. The flowering time and leaf number of *Col*, transgenic *fwa-d|35S::FWA-inverted-repeat,* and untransformed *fwa-d* plants were then recorded and compared using a T-test. All plants were

grown under 16 hours light/8 hours dark conditionas at 22 degrees Celsius. Rosette leaf numbers were then counted as the first flower on the individual plant fully opened. As before, flowering time and leaf number were used as a proxy for RdDM of the *FWA* locus.

Chapter 3

Results

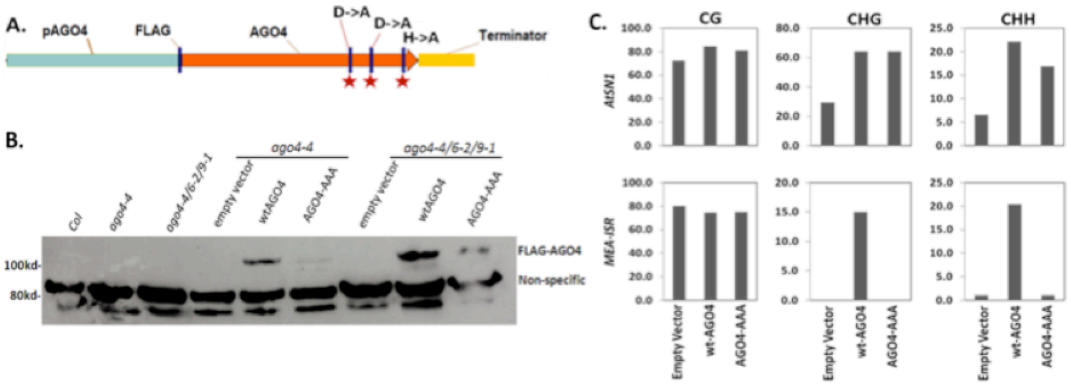

Figure 2: Preliminary results with AGO4 constructs. A: Schematic of AGO4-AAA construct, with the promoter, FLAG tag, Ago gene, and terminator labeled. Starred lines indicated the amino acids in the slicing domain changed to alanine. B: Western blot for FLAG-AGO4. The first three columns are untransformed controls, while the subsequent columns show an empty vector, a vector with wild type AGO4, and a vector with the AGO4-AAA construct respectively transformed into both the *ago4-4* and the *ago4-4/6-2/9-1* mutant. C: Methylation profile of transformants. Percent CG, CHG, and CHH methylation at two loci shown for each of the *ago4-1/6-2/9-1* transformants.

Figure 2A shows a schematic of the AGO4-AAA construct, with the stars indicating the three critical amino acids changed to alanine. In the western blot in figure 2B, we see that there is accumulation of the 100kd FLAG-AGO4 band in the mutants transformed with the wtAGO4 construct, and to some degree with the AGO4-AAA construct, but not in empty vector transformed or untransformed samples. This indicates that the AGO4-AAA construct is expressed in the transformants, although it is not clear to what degree the AGO4-AAA accumulates. Figure 2C shows the methylation profile of the *ago4-4* transformants at the *MEA-ISR* and *ATSN1* loci. CHH and some CHG methylation was lost in the empty vector transformants, indicating a lack of RdDM. This loss of methylation was complemented by the wt-AGO4 transformants. However, the AGO4-AAA slicing deficient transformants did not complement the methylation loss to the same degree, implying slicing activity is necessary for full AGO4 function.

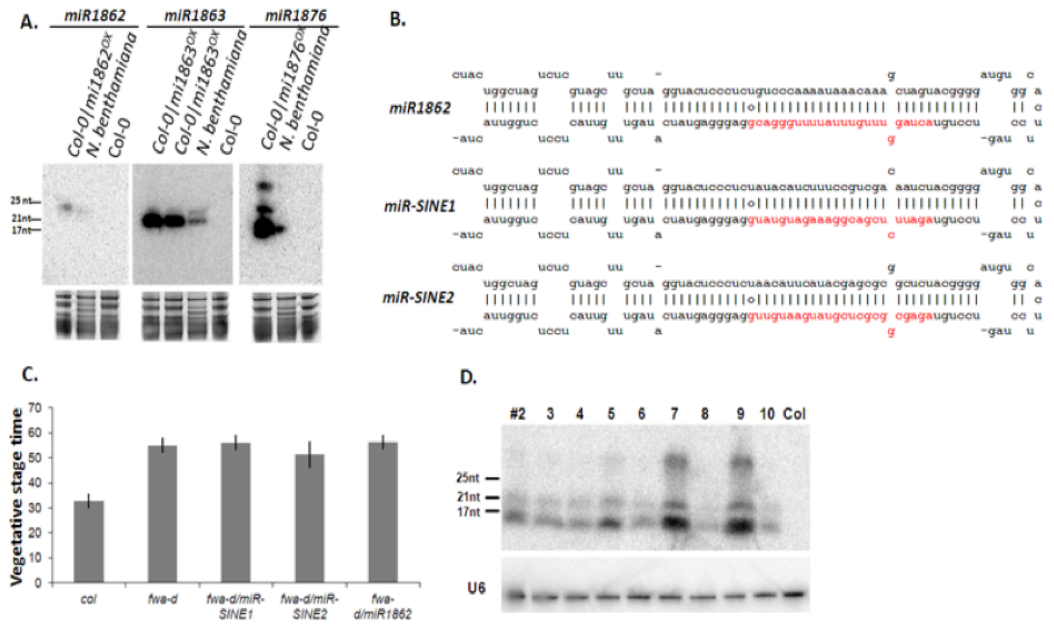

Figure 3: 24nt Rice miRNA Expression in *Arabidopsis.* A: Northern Blot for 24nt miRNA expression, with transformed *Arabidopsis*, infiltrated *N. benthamiana*, and control miRNA fragment size accumulations tested for each of the three 24nt miRNAs. B: Schematic of artificial 24nt miR design, with red portion showing the 24nt miRNA expected to be produced from each stem-loop structure. C: Flowering time of miRNA transformants compared with untransformed *col* and *fwa-d* controls. D: Northern Blot of miR-SINE2 expression, showing accumulation of 24nt miR-SINE2 RNAs.

Figure 3A shows the RNA blot of transformed *Arabidopsis*, infiltrated *Nicotiana benthamiana*, and a control for three different 24nt rice miRNA constructs. The blots for miR1863 and miR1876 show a variety of bands, but none at 24 nucleotides. Faint 24nt bands were visible for miR1862, so it was used as the basis for the two miR constructs targeting FWA seen in figure 3B. However, none of the transformants seemed to alter the flowering time phenotype in *fwa-d* plants, as shown in figure 3C. Since the *fwa-d*/*miR-SINE2* transformants seemed like it might have had an altered flowering time phenotype, a northern blot was done to assess miR-SINE2 accumulation. No 24nt RNA was detected, meaning the failure of the 24nt miR constructs to rescue the *fwa-d* late flowering phenotype was due to lack of accumulation. No conclusions can therefore be drawn on the efficacy of 24nt miR on RdDM in *Arabidopsis*.

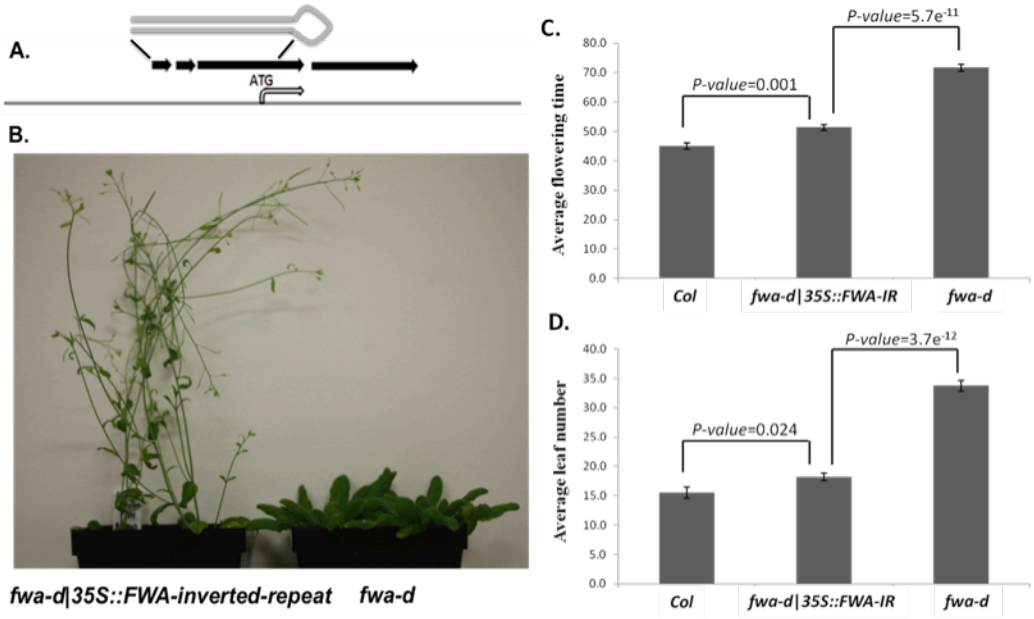

Figure 4: irRNA for *FWA* tandem repeat region rescues *fwa-d* phenotype. A: Schematic of irRNA. B: Phenotype of irRNA Transformed and Untransformed *fwa-d Arabidopsis*. C: Flowering Time of *fwa-d*/35S::FWA-IR Plants and Controls. Leaf number of *fwa-d*/35S::FWA-IR Plants and Controls.

Figure 4A shows the structure of the gray irRNA stem-loop, with the bottom line indicating the *FWA* locus, black arrows indicating tandem repeat regions in the *FWA* locus, and the black lines showing the area of the locus covered by the irRNA. Figure 4B shows the dramatic phenotype change induced by the transgene in *fwa-d* plants, with *35S::FWA-inverted-repeat* transformed plants regaining an earlier flowering, spindlier phenotype than *fwa-d* controls. Figure 4C quantifies the difference in flowering time, and figure 4D in average leaf number. The *fwa-d/35S::FWA-IR plants* were slightly but statistically significantly later flowering and had higher leaf number than Columbia controls, with p-values of .001 and .024 respectively. However, the difference was much greater between the transformants and the untransformed *fwa-d* plants, with dramatically different values for flowering time and leaf number, and statistically significant p-values less than 10^{-10}. This is a strong demonstration that the 35S::FWA-inverted-repeat transgene is effective in rescuing the *fwa-d* phenotype.

Chapter 4

Conclusion

The first set of experiments sought to use the slicing deficient AGO4-AAA transgene to demonstrate the impact of AGO4 slicing activity on RdDM. However, these preliminary AGO4 slicing experiments are inconclusive due to limited AGO4-AAA accumulation (Figure 2B). Though the AGO4-AAA transformed mutants had an incomplete phenotype rescue (Figure 2C), which seemingly supports the hypothesis that slicing activity is necessary for complete AGO4 function, the very limited AGO4-AAA accumulation opens the possibility that the limited AGO4-AAA function may merely be due to lack of quantity, not efficiency. Because no conclusion can be drawn from the current data, future experiments will have to employ a different strategy, such as an AGO4 slicing mutant with higher accumulation.

The second set of experiments tested the hypothesis that artificial miRNAs based on rice 24nt miRNAs could be used to induce targeted RdDM. These preliminary experiments with 24nt miRNAs are not promising for future research in this direction. Only one of the three miRNAs investigated had significant 24nt accumulation in a transgenic system (Figure 3A), and the targeted miRNAs designed from it did not accumulate once transformed into *Arabidopsis* (Figures 3C and 3D). It is not known if this is due to a problem with the miRNA design, or a problem with production or stability of 24nt miRNAs in *Arabidopsis*. Regardless, the 24nt miRNA system does not appear to be currently viable for investigation of siRNA matching requirements in RdDM.

The final set of experiments, testing whether irRNA could be used to induce RdDM, did support the hypothesis. Though not as precise as the miRNA system would have been, the irRNA

system does result in almost complete rescue of the *fwa-d* phenotype (Figure 4). Though the irRNA transgene produces a variety of RNA fragments, it can be ensured that they all have an equal degree of overall complementarity to the target locus by spacing mismatches evenly throughout the stem-loop. For instance, if a single mismatch is placed every 24 nucleotides, then all resulting 24nt RNA fragments will have exactly one mismatch, although its position will be random. Despite this randomness, the irRNA system will enable future experiments to elucidate the overall degree of complementarity necessary for RdDM

Going forward, a new, more stable slicing-deficient AGO4 transgene will have to be created to test the importance of AGO4 slicing in RdDM. Altering only one of the critical slicing residues, rather than all three could do this. If this still resulted in lower protein accumulation than the unaltered AGO4 transgene, the effects of AGO4 accumulation on RdDM would have to be accounted for. This could be done by quantifying the accumulation of each and mathematically estimating what portion of the differences in RdDM were due to differences in abundance. Another way of doing this could be to lower the accumulation of the unaltered AGO4 so that it was comparable to the slicing deficient version.

As for the study of the sequence complementarity requirements for RdDM, a variety of irRNAs with various degrees of complementarity to the *FWA* locus can be generated to test this. A further point of interest for future study is to test the effects of both the irRNA and AGO4 transgenes on RdDM at other loci. This would enable comparison of the consistency of these effects, enabling generalization to other genes. Further generalization would be enabled by testing the requirements for RdDM in other organisms, including plants and other phyla.

Hopefully, this and future experiments will enable future biotechnology in RdDM. For instance, knowing the specific sequence requirements could enable heritable artificial RdDM of targeted loci in plants, without methylation at unforeseen loci. Perhaps RdDM could even be altered in intensity by altering the sequence complementarity. Such technologies would enable

promotion of desirable traits in crop plants, as well as enabling future work in plant biology research. By investigating RdDM, agriculture and knowledge of basic biology will ultimately benefit.

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ACADEMIC VITA

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Education

B.S., Biology, 2013, The Pennsylvania State University, University Park, PA

DMD, 2018 (expected), The University of Pittsburgh

Honors and Awards

Dean's List, Penn State, each semester Fall 2010-Fall 2013

Braddock Scholarship, Eberly College of Science at Penn State, 2010

Academic Excellence Scholarship, Schreyer Honors College at Penn State, 2010

Activities

Running

- *Member of Penn State Club Cross Country*
- *Qualified for 2014 Boston Marathon*

FISH Youth Group Leader

- *Helped plan and direct weekly activities for up to 70 high schoolers*
- *Helped lead Spring Break service trips to Pittsburgh, PA*

Travel

- *Bicycled 3,700 miles from Virginia to San Francisco*
- *Traveled to Costa Rica for Tropical Field Ecology class over Winter Break, 2013*

Research Experience

Penn State Department of Biology: Axtell Lap Group, Fall 2011-Present

- *Performed molecular biology techniques including PCR and plant and bacterial transformation*
- *Prepared and analyzed samples for genetic sequencing*
- *Assisted graduate students in the lab*

Penn State Department of Chemical Engineering, Curtis Lab Group, Summer 2011

- *Assisted graduate students in the lab*
- *Learned skills including PCR, gel electrophoresis and aseptic technique*

Professional Experience

Dental Shadowing

- *Shadowed 8 dentists in State College for over 60 hours total*