A Null Allele of the Pol IV Second Subunit is Viable in *Oryza sativa*

Tania Chakraborty¹, Joshua T. Trujillo², Timmy Kendall¹, and Rebecca A. Mosher¹

¹ School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

² Department of Biochemistry, Purdue University, West Lafayette, IN 47907, USA

Corresponding author: Rebecca A. Mosher, rmosher@email.arizona.edu

Keywords: RNA-directed DNA Methylation, RNA Polymerase IV, *Oryza sativa*, small RNA, DNA methylation

ABSTRACT

All eukaryotes possess three DNA-dependent RNA polymerases, Pols I-III, while land plants possess two additional polymerases, Pol IV and Pol V. Derived through duplication of Pol II subunits, Pol IV produces 24-nt siRNAs that interact with Pol V transcripts to target *de novo* DNA methylation and silence transcription of transposons. Members of the grass family encode additional duplicated subunits of Pol IV and V, raising questions regarding the function of each paralog. In this study, we identify a null allele of the putative Pol IV second subunit, *NRPD2*, and demonstrate that NRPD2 is the sole subunit functioning with NRPD1 in small RNA production and CHH methylation in leaves. Homozygous *nrpd2* mutants have neither gametophytic defects, nor embryo lethality, although adult plants are dwarf and sterile.

INTRODUCTION

Plant genomes are continuously under threat from transposons, mobile genetic elements whose transposition causes mutation (Dubin, Mittelsten Scheid, and Becker 2018). Host cells use DNA and histone methylation to suppress transposon activity and keep the damage to a minimum. In heterochromatin, transposons are silenced through dense CG methylation and a self-reinforcing loop of non-CG methylation and di-methylation of Histone H3 Lysine 9 (Zemach et al. 2013; Stroud et al. 2014; Du et al. 2012). In gene-proximal regions, transposons are silenced by RNA-directed DNA Methylation (RdDM) (Q. Li et al. 2015; Zhong et al. 2012). Although RdDM can *de novo* methylate cytosines in CG, CHG and CHH contexts (where H is any base other than G), maintenance of CG and CHG methylation by other pathways means that CHH methylation is a primary indication of RdDM (Stroud et al. 2014).

During RdDM, RNA Polymerase (Pol) IV produces short single-stranded RNA transcripts which are simultaneously transcribed into double-stranded RNA by RNA DEPENDENT RNA POLYMERASE 2 (RDR2) (Blevins et al. 2015; Zhai et al. 2015; Singh et al. 2019). These double-stranded RNAs are chopped into 24-nt short interfering (si)RNAs by DICER-LIKE 3 (DCL3), and one of the duplexed siRNAs is loaded into ARGONAUTE 4 (AGO4) (Xie et al. 2004; Kasschau et al. 2007; Singh et al. 2019; Mi et al. 2008; Havecker et al. 2010). AGO4 associates with the carboxy-terminal domain of RNA Pol V, and base-pairing of the nascent Pol V transcript and siRNA recruits DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which causes *de*

novo cytosine methylation (C. F. Li et al. 2006; El-Shami et al. 2007; Wierzbicki et al. 2009; Cao et al. 2003).

Pol IV and V are plant-specific polymerases, which evolved through duplication and functional divergence of multiple Pol II subunits (Luo and Hall 2007; Y. Wang and Ma 2015; Tucker et al. 2010; Huang et al. 2015). Many subunits are shared by Pol II, Pol IV, and Pol V, while unique subunits are presumed to confer the specialized activities of these polymerases (Ream et al. 2009; Haag et al. 2014). In Arabidopsis, the largest subunits (NRPD1 and NRPE1 for Pol IV and V, respectively), are unique, while the second subunit (NRPD2) is shared by Pol IV and Pol V (Ream et al. 2009; Haag et al. 2014). Together, these two largest subunits form the catalytic center of the polymerase. In the grass family, *NRPE1* and *NRPD2* duplicated, giving rise to *NRPF1* and *NRPF2*, respectively (Trujillo et al. 2018). Phylogenetic analysis suggests that these paralogs have new functions, however their functions are not clear.

Mutation of *NRPD1* causes a near-complete loss of 24-nt siRNAs, while disruption of *NRPE1* causes only a small decrease in 24-nt siRNA accumulation (Mosher et al. 2008; Gouil and Baulcombe 2016; Grover et al. 2018; Erhard et al. 2009; Z. Wang et al. 2020; Xu et al. 2020; Debladis et al. 2020; Zheng et al. 2021). However, because both Pol IV and Pol V are required for RdDM, CHH methylation is reduced when either largest subunit is eliminated (Gouil and Baulcombe 2016; Stroud et al. 2013; Zheng et al. 2021). Although Arabidopsis *nRPD1* or *nRPE1* mutants develop normally, loss of *NRPD1* or *NRPE1* in many other species causes developmental defects (Hollick 2010; Gouil and Baulcombe 2016; Grover et al. 2018; Xu et al. 2020; C. Zhang et al. 2020; Z. Wang et al. 2020; Zheng et al. 2021).

Relative to the unique largest subunits, the shared second subunit of Pol IV and V is less studied. Arabidopsis *nRPD2* mutants lack 24-nt siRNAs and CHH methylation and do not accumulate Pol V transcripts (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005; X. Zhang et al. 2007; Wierzbicki, Haag, and Pikaard 2008). Like mutations of the largest subunits, *nRPD2* plants do not exhibit developmental phenotypes in Arabidopsis. In maize, there are three second subunit paralogs (two copies of NRPD2 and one copy of NRPF2) and phylogenetic analysis suggests these paralogs are functionally distinct (Haag et al. 2014; Trujillo et al. 2018). Mutants of *NRPD2a* show disrupted 24-nt siRNA production and defective paramutation, suggesting that there is little redundancy between paralogs (Sidorenko et al. 2009; Stonaker et al. 2009), while immunoprecipitation of polymerase complexes also hints that the duplicated NRPD2 subunits in grasses are not functionally redundant (Haag et al. 2014; Trujillo et al. 2018).

RdDM is critical for development in rice. Loss of *NRPD1* causes sterility, reduced plant height, and increased tillering (Xu et al. 2020; C. Zhang et al. 2020; Zheng et al. 2021). Mutations in other components of the RdDM pathway in rice have similar developmental phenotypes. *dcl3* RNAi lines showed reduced plant height and increased flag leaf angle, along with reduced panicle size (Wei et al. 2014). *ago4ab* and *rdr2* RNAi lines also showed reduced height and panicle size, and the *ago4ab* line phenocopied *dcl3* in flag leaf angle (Wei et al. 2014). *drm2* mutants showed a wide range of developmental phenotypes including complete sterility (Moritoh et al. 2012).

In this study, we characterize a rice T-DNA insertion mutant in *NRPD2*, the gene presumed to encode the second subunit for both Pol IV and Pol V. We show that the *nRPD2* mutant loses nearly all 24-nt siRNAs, especially from TE-rich regions, and also loses CHH methylation in

specific sites. These molecular and developmental phenotypes largely copy *nrpd1* mutants, indicating that *NRPD2* is the sole second subunit for Pol IV in leaves.

RESULTS

Rice *NRPD2* is required for 24-nt siRNA production

To investigate the function of *NRPD2* in rice, we searched the PFG T-DNA insertion library and obtained a line with an insertion in the Rpb6 domain (**Figure 1A**) (Jeon et al. 2000; Jeong et al. 2006). RT-PCR confirmed the loss of *NRPD2* gene expression in plants homozygous for the mutation (**Figure 1B**). Sequencing wild-type small RNA populations confirmed that 24-nt small RNAs are the plurality of total small RNAs in *Oryza sativa* cv. Japonica var. Hwayoung, totaling 46% of small RNAs in leaves. In comparison to wild type, *nrpd2* mutants show ~69% reduction of 24-nt siRNAs (**Figure 1C**). This reduction is consistent with *nrpd2* mutants in Arabidopsis (X. Zhang et al. 2007), and *nrpd1* mutants in rice (Xu et al. 2020; Debladis et al. 2020; Zheng et al. 2021). 21- and 22-nt sRNAs are elevated in *nrpd2* populations, most likely due to oversampling of these sizes following depletion of the dominant 24-nt size class. Together, these results show that *NRPD2* is required for majority of the 24-nt small RNA production in rice leaves.

NRPD2 function overlaps sites of *NRPD1* action

To directly compare the function of *NRPD2* with *NRPD1*, we obtained sRNA sequencing data from rice *nrpd1a nrpd1b* double mutants (*nrpd1*, hereafter) (Zheng et al. 2021) and used the 300-bp non-overlapping windows to count small RNAs. There was a good correlation between wild-type sRNA accumulation in leaf and seedling tissues (**Figure 2A**). Because there is 24-nt siRNA depletion in both *nrpd2* and *nrpd1* mutants, we wanted to understand if this loss was distributed across many genomic loci, or if it came from a small subset of abundantly-expressed loci (Burgess et al. 2021). To examine this, we assessed sRNA accumulation in 300-bp windows. Among windows with at least 5 mapped reads in wild type in any size class, those with less than 10% of wild-type sRNA accumulation were called “depleted windows”, while windows showing an increase in sRNA accumulation compared to wild type were called “independent windows”. We

observed that in the *nrpd2* mutant there are 188,215 depleted windows (~78%), indicating that the loss of small RNAs observed in the mutant occurs at most small RNA-producing loci. We observed a similar percentage of siRNA-depleted windows (~77%) in *nrpd1* (**Figure 2B**).

To better understand the genomic regions where NRPD2 acts, we examined overlaps between *nrpd2*-depleted or -enriched windows and genomic features like genes, transposons, miRNAs, centromeric and telomeric repeats. We observed that the *nrpd2*-depleted windows were significantly enriched for DNA transposons, MITE elements, and SINE retrotransposons, but were depleted in genes and LTR retrotransposons (**Table 1**). This pattern is similar to regions enriched or depleted at sites of RdDM in *Brassica rapa* (Grover et al. 2018), indicating that *nrpd2*-depleted windows resemble canonical RdDM loci. The *nrpd2*-independent windows overlapped significantly with genes, miRNAs, and short transposons (MITEs and SINEs). To understand if the overlap of *nrpd2*-independent windows with MITEs was because of the physical proximity of the MITEs and SINEs to genes, which accumulate *nrpd2*-independent small RNAs, we calculated the distance between genes and the MITEs and SINEs that overlap the *nrpd2*-depleted and *nrpd2*-independent windows. We observed that while most *nrpd2*-depleted windows that overlap MITEs and SINEs are far away from genes, majority of *nrpd2*-independent windows that overlap MITEs and SINEs also overlap genes (**Supplemental Figure 1**). These observations demonstrate that *nrpd2*-depleted windows resemble canonical RdDM loci.

To investigate whether *nrpd2* affected the same genomic regions as a loss of *nrpd1*, we overlapped the small RNA depleted windows from both mutants. We observed a significant overlap between the two sets of genomic locations (**Figure 2C**) and a similar enrichment of SINE and MITE elements in *nrpd1*-independent windows, indicating that NRPD1 and NRPD2 are required in the same locations.

Together, these data indicate that *nrpd2* phenocopies *nrpd1* in rice leaf, suggesting that NRPD2 is the sole second subunit assembled in Pol IV in leaves.

Table 1: Enrichment of genomic features across *nrpd2*-depleted and *nrpd2*-independent windows

Feature	Fold change	
	<i>nrpd2</i> -depleted windows	<i>nrpd2</i> -independent windows
DNA Transposons	1.9***	n.s.
MITE Transposons	2.3***	2.1***
LTR Transposons	0.52***	0.73***
LINE Transposons	0.83*	0.24**
SINE Transposons	9.6***	7.4***
Telomere repeats	16.5***	n.s.
Genes	0.44***	5.2***
lmiRNAs	n.s.	61.1**
miRNAs	0.10***	25.3***
Centromeric repeats	n.s.	n.s.

*, $p < 0.05$, **, $p < 10^{-4}$, ***, $p < 10^{-10}$

Rice NRPD2 participates in RdDM

To confirm that the siRNAs produced by NRPD2 act in RdDM, we assayed methylation with quantitative Chop-PCR at loci producing *nrpd2*-dependent 24-nt siRNAs (H. Zhang et al. 2014). Loci were randomly selected based on the presence of an *Nla III* site, at least 90% reduction in 24-nt siRNA accumulation in the *nrpd2* mutant, and low variability in qPCR. All 15 of the windows showed higher methylation in wild type than *nrpd2*, although not all changes

are significant and sometimes methylation in wild type was low, perhaps as a consequence of the assay testing methylation at a single cytosine (**Supplemental Figure 2**). Of the 8 windows with at least 5% methylation in WT, 7 were substantially reduced in *nrpd2* (**Figure 3**). These results indicate that CHH methylation at *nrpd2*-depleted windows requires NRPD2.

nrpd2 mutants are viable but infertile

Rice lacking Pol IV or Pol V function show reduced height and drastic loss in fertility (Zheng et al. 2021). To investigate the morphological phenotypes of the *nrpd2* mutant, we grew selfed seeds from a heterozygous plant and observed 11 wild type, 23 heterozygotes, and 9 homozygous mutants, indicating there were neither gametophytic nor embryonic defects associated with loss of NRPD2 function. Heterozygous *nrpd2* mutants were indistinguishable from their wild-type siblings, however homozygous *nrpd2* mutants were substantially shorter (**Figure 4A**). The homozygous *nrpd2* mutants also produced fewer tillers than their wild-type and heterozygous siblings (**Figure 4B**), in contrast to *nrpd1*, which is reported to increase tiller number (Xu et al. 2020). This difference might be due to different genetic backgrounds, as the *nrpd1* mutation is in the Japonica cultivar Nipponbare, while the *nrpd2* mutation

Figure 3. NRPD2 is required for RdDM. Quantitative methylation-sensitive PCR (chop-qPCR) for CHH methylation at *nrpd2*-depleted windows demonstrates that NRPD2 is required for RdDM. The mean and standard deviation of four independent biological replicates are shown in bars. Individual replicates are shown as black dots.

is in the Japonica cultivar Hwayoung. Although homozygous *nrpd2* mutants produced panicles, these were small and frequently did not emerge from the sheath (**Figure 4C, D**). Florets on these panicles were misshapen and never produced viable seed, indicating that *NRPD2* function is essential for reproductive development.

DISCUSSION

RNA-directed DNA methylation silences euchromatic transposons, potentially influencing the expression of nearby genes (Dubin, Mittelsten Scheid, and Becker 2018). Although the rice genome is smaller than many flowering plants, approximately half of its genome is transposons, including a large number of MITEs (Minature Inverted repeat Transposable Elements) near genes (Jiang and Panaud 2013; Song and Cao 2017). Thus, RdDM is expected to be important both for transposon control and gene expression in rice. As rice is one of the important food crops across the world, it is therefore essential to understand the mechanism of RdDM in rice. We characterized a T-DNA insertional mutation in *Oryza sativa* *NRPD2*, the putative second subunit of RNA Pol IV and Pol V, and provided evidence that this subunit is required for Pol IV function.

Poaceae-specific duplication of putative Pol V largest (NRPE1) and second-largest (NRPD2) subunits raises interesting questions about the assembly of multiple non-canonical polymerases in rice (Trujillo et al. 2018). Zheng and colleagues recently demonstrated that NRPF1 is not redundant with NRPE1, and not required for RdDM in seedlings (Zheng et al. 2021), suggesting that each paralogous largest subunit has unique function. However, sharing of the second subunit between Arabidopsis Pol IV and Pol V suggests that Pol function is primarily determined by the largest subunit and that second subunits might be redundant. We have shown that *nrpd2* mimics *nrpd1* with respect to small RNA accumulation, DNA methylation, and

developmental phenotype. We therefore conclude that NRPD2 is the sole second subunit assembled in Pol IV and is not redundant with NRPF2. While our molecular analysis is limited to Pol IV activity in leaves, shared developmental phenotypes indicate that NRPD1 and NRPD2 function together exclusively throughout rice development.

NRPD2 targets are enriched for transposons, including MITEs, which are frequently found in proximity to genes in rice (Jiang and Panaud 2013). Loss of methylation at MITEs in the *nRPD1* mutant causes increased expression of a miRNA gene and downregulation of its target protein-coding gene, ultimately causing increased tillering (Xu et al. 2020). We did not observe increased tillering in *nRPD2* mutants; indeed, homozygous mutants have fewer tillers than wild-type or heterozygous siblings, and more closely resemble knockdown mutants of *dcl3* or *drm2* (Moritoh et al. 2012; Wei et al. 2014). Embryonic lethality was also reported for *nRPD1* mutants, however, we observe homozygous mutant seeds arising at the expected ratios, indicating there is no lethality for homozygous mutant embryos. *NRPE1* mutations in different rice strains result in different phenotypes (Zheng et al. 2021), suggesting that genomic differences between rice strains, especially with respect to transposon insertion site, might explain the differences between *nRPD1* and *nRPD2* phenotypes we observed.

A phenotype shared by all of the various rice RdDM mutations is reduced fertility (Moritoh et al. 2012; Wei et al. 2014; Xu et al. 2020; Zheng et al. 2021). RdDM is also required for full fertility in a range of other angiosperms (Chow, Chakraborty, and Mosher 2020), suggesting that defects in reproduction are deeply conserved and less likely to result from transposon-mediated regulation of individual genes. Given the importance of rice reproduction for the global food supply, understanding how genes like *NRPD2* contribute to successful reproduction is a critical area of research.

METHODS

Plant Material and Growth Conditions

The PFG T-DNA allele of *NRPD2* (PFG_1A-24731.R) is in the *Oryza sativa* (Japonica) Hwayoung background. All plants were grown in a greenhouse at 85°F and were genotyped with primers flanking the T-DNA insertion site and T-DNA border primers (**Supplemental Table 1**). Wild type amplifies a product only with the flanking primers, while homozygous *nRPD2* mutants amplify products with a combination of flanking and border primers. Heterozygous mutants amplify products with both combinations of primers. Homozygous plants and wild-type sibling controls were derived from self-fertilized heterozygous parents.

Small RNA sequencing

Leaf tissue was collected from three wild type and three homozygous *nRPD2* mutant siblings and total nucleic acid was prepared (White and Kaper 1989). The small RNA (<200 nt) fraction was purified with the mirVana miRNA isolation kit (ThermoFisher) prior to small RNA sequencing library preparation using the NEBNext Small RNA kit (NEB; E7330). Libraries were pooled and sequenced by The University of Arizona Genetics Core on an Illumina NextSeq500. These datasets are available in the National Center for Biotechnology Information Sequence Read Archive under Bio Project PRJNA758109.

Small RNA analysis

FastQC (Andrews 2010) was used to quality-check the raw small RNA reads that were obtained from the sequencing facility. Adapters were removed with Trim Galore, with options -length 10 and -quality 20 (Krueger v0.6.5 2019). After that, reads mapping to the *Oryza sativa*, *Brassica rapa*, and *Arabidopsis thaliana* chloroplast and mitochondrial genomes were removed by aligning to these genomes using Bowtie (Langmead et al. 2009). Reads mapping to structural and noncoding RNAs were also removed by aligning to the rfam database (Kalvari et al. 2018) using all available *Oryza sativa* sequences. After this, reads at least 19-nt and up to 26-nt were selected and aligned to the *Oryza sativa* var. Japonica genome using Bowtie. Only sRNA reads with a perfect genomic match were kept for later analysis. ShortStack (Axtell 2013) was used to annotate all small RNA loci, with options -mismatches 0, -mmap u, -mincov 0.5 rpm, and -pad 75. All of these steps can be done with a small RNA analysis pipeline (Bose 2019). ShortStack was used again to count small RNAs on 300-bp tiled windows across the genome. Upon investigation of the windows, it was observed that four windows produced a large number of small RNAs, almost to a tenth of the whole library size. Upon a BLAST search, we observed that most of the small RNAs mapped to rRNAs in the Rfam database, and therefore we removed all the reads from these four windows. The reads were normalized to reads per million mapped reads (RPM) and all further analysis were performed on normalized reads. The number of reads at each step in each replicate can be found in **Supplemental Table 2**.

Principal component analysis was done on siRNA accumulation in each 300-bp window to check for consistency of the replicates (**Supplemental Figure 3**), and size profiles of mapped small RNAs in the replicates was used as an additional check.

To measure the impact of the *nprpd2* mutation, only windows whose expression across 19-26mers was at least 0.5 RPM in both our leaf wild-type and seedling wild-type (Zheng et al. 2021) samples were considered for further analysis. *nprpd2*-depleted and *nprpd2*-independent windows were overlapped with genomic features using “bedtools overlap” function (Quinlan and Hall 2010) and overlaps of at least 1 nt were counted. Expected values were calculated based on the number of 300-nt tiled windows containing the genomic features and Fisher’s exact tests were performed to understand the significance of enrichment or depletion. Only significant overlaps are reported.

For the correlation plot, we plotted normalized small RNAs across windows in both wild-type samples. For the Venn diagram, we obtained the list of “*nprpd2*-depleted” and “*nprpd1*-depleted” windows and calculated the overlap between those with “bedtools overlap” function. Then we used the Venn Diagram Plotter (Kyle Littlefield and Matthew Monroe 2020).

RT-PCR and Chop-qPCR for methylation analysis

For RT-PCR of *ACTIN* and *NRPD2*, total nucleic acid was prepared as described above and treated with DNase using the DNA-free DNA Removal Kit (ThermoFisher). A One-step RT-qPCR kit (Promega) was used for converting the RNA into cDNA before amplifying target transcripts on a CFX Connect Real-Time PCR Detection System (Biorad). RT-PCR primers are listed in **Supplemental Table 1**.

For Chop-qPCR, we gathered the genomic sequence of windows where *nprpd2* and *nprpd1* showed at least 90% reduction in 24-nt siRNAs compared to wild type and an *Nla III* recognition sequence (CATG) was present. *Nla III* is sensitive to methylation of the first C in its recognition sequence, a CHH site. We digested 1 µg of wild type or *nprpd2* DNA with *Nla III* and also performed

a “mock” digestion without enzyme. Then we used the digested and mock treated samples to amplify these twenty-five loci as well as *ACTIN* as a control. Amplification was quantified with a CFX Connect Real-Time PCR Detection System (Biorad) qPCR machine.

Relative percentage methylation at each site was calculated by comparing the Cq values for digested and mock digested treatments for each sample (H. Zhang et al. 2014). Chop-qPCR primers are listed in **Supplemental Table 1**.

Supplemental Information

Supplemental Figures 1-3, Supplemental Table 1-2

Authors' contributions

RAM conceptualized the project; TC, TK, and JT performed experiments; TC and RAM analyzed and visualized the data and wrote the paper.

Acknowledgements

The authors gratefully acknowledge support from the National Science Foundation (MCB-1929678 to RAM). The authors would also like to extend gratitude to Dr. Jesse Woodson for use of his qPCR machine, and Ms. Kyungsook An for sending the PFG T-DNA lines. Illumina sequencing was performed by The University of Arizona Genetics Core.

References

- Andrews, Simon. 2010. “FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available Online at: <http://www.Bioinformatics.Babraham.Ac.Uk/Projects/Fastqc/>.” 2010. <https://github.com/s-andrews/FastQC>.
- Axtell, Michael J. 2013. “ShortStack: Comprehensive Annotation and Quantification of Small RNA Genes.” *RNA* 19 (6): 740–51.
- Blevins, Todd, Ram Podicheti, Vibhor Mishra, Michelle Marasco, Jing Wang, Doug Rusch, Haixu Tang, and Craig S. Pikaard. 2015. “Identification of Pol IV and RDR2-Dependent Precursors of 24 Nt siRNAs Guiding de Novo DNA Methylation in Arabidopsis.” *ELife* 4 (OCTOBER2015). <https://doi.org/10.7554/eLife.09591>.
- Bose, Maya. 2019. “SRNA_snakemake_workflow: A Snakemake Workflow for an SRNA-Seq Data Analysis Pipeline Developed by the Mosher Lab.” November 2019. https://github.com/boseHere/sRNA_snakemake_workflow.
- Burgess, Diane, Hiu Tung Chow, Jeffrey W. Grover, Michael Freeling, and Rebecca A. Mosher. 2021. “Ovule siRNAs Methylate and Silence Protein-Coding Genes in Trans.” *BioRxiv*. <https://doi.org/10.1101/2021.06.10.447945>.
- Cao, Xiaofeng, Werner Aufsatz, Daniel Zilberman, M. Florian Mette, Michael S. Huang, Marjori Matzke, and Steven E. Jacobsen. 2003. “Role of the DRM and CMT3 Methyltransferases in RNA-Directed DNA Methylation.” *Current Biology: CB* 13 (24): 2212–17.

- Chow, Hiu Tung, Tania Chakraborty, and Rebecca A. Mosher. 2020. "RNA-Directed DNA Methylation and Sexual Reproduction: Expanding beyond the Seed." *Current Opinion in Plant Biology*. Elsevier Ltd. <https://doi.org/10.1016/j.pbi.2019.11.006>.
- Debladis, Emilie, Tzuu-Fen Lee, Yan-Jiun Huang, Jui-Hsien Lu, Sandra M. Mathioni, Marie-Christine Carpentier, Christel Llauro, et al. 2020. "Construction and Characterization of a Knock-down RNA Interference Line of OsNRPD1 in Rice (*Oryza Sativa* Ssp Japonica Cv Nipponbare)." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 375 (1795): 20190338.
- Du, Jiamu, Xuehua Zhong, Yana V. Bernatavichute, Hume Stroud, Suhua Feng, Elena Caro, Ajay A. Vashisht, et al. 2012. "Dual Binding of Chromomethylase Domains to H3K9me2-Containing Nucleosomes Directs DNA Methylation in Plants." *Cell* 151 (1): 167–80.
- Dubin, Manu J., Ortrun Mittelsten Scheid, and Claude Becker. 2018. "Transposons: A Blessing Curse." *Current Opinion in Plant Biology* 42 (April): 23–29.
- El-Shami, Mahmoud, Dominique Pontier, Sylvie Lahmy, Laurence Braun, Claire Picart, Danielle Vega, Mohamed-Ali Hakimi, Steven E. Jacobsen, Richard Cooke, and Thierry Lagrange. 2007. "Reiterated WG/GW Motifs Form Functionally and Evolutionarily Conserved ARGONAUTE-Binding Platforms in RNAi-Related Components." *Genes & Development* 21 (20): 2539–44.
- Erhard, Karl F., Jr, Jennifer L. Stonaker, Susan E. Parkinson, Jana P. Lim, Christopher J. Hale, and Jay B. Hollick. 2009. "RNA Polymerase IV Functions in Paramutation in *Zea Mays*." *Science* 323 (5918): 1201–5.
- Gouil, Quentin, and David C. Baulcombe. 2016. "DNA Methylation Signatures of the Plant Chromomethyltransferases." Edited by Ortrun Mittelsten Scheid. *PLoS Genetics* 12 (12): e1006526.
- Grover, Jeffrey W., Timmy Kendall, Abdul Baten, Diane Burgess, Michael Freeling, Graham J. King, and Rebecca A. Mosher. 2018. "Maternal Components of RNA-directed DNA Methylation Are Required for Seed Development in *Brassica Rapa*." *The Plant Journal: For Cell and Molecular Biology* 94 (4): 575–82.
- Haag, Jeremy R., Brent Brower-Toland, Elysia K. Krieger, Lyudmila Sidorenko, Carrie D. Nicora, Angela D. Norbeck, Andre Irsigler, et al. 2014. "Functional Diversification of Maize RNA Polymerase IV and V Subtypes via Alternative Catalytic Subunits." *Cell Reports* 9 (1): 378–90.
- Havecker, Ericka R., Laura M. Wallbridge, Thomas J. Hardcastle, Maxwell S. Bush, Krystyna A. Kelly, Ruth M. Dunn, Frank Schwach, John H. Doonan, and David C. Baulcombe. 2010. "The Arabidopsis RNA-Directed DNA Methylation Argonautes Functionally Diverge Based on Their Expression and Interaction with Target Loci." *The Plant Cell* 22 (2): 321–34.
- Herr, A. J., M. B. Jensen, T. Dalmay, and D. C. Baulcombe. 2005. "RNA Polymerase IV Directs Silencing of Endogenous DNA." *Science* 308 (5718): 118–20.
- Hollick, Jay B. 2010. "Paramutation and Development." *Annual Review of Cell and Developmental Biology* 26 (1): 557–79.
- Huang, Yi, Timmy Kendall, Evan S. Forsythe, Ana Dorantes-Acosta, Shaofang Li, Juan Caballero-Pérez, Xuemei Chen, Mario Arteaga-Vázquez, Mark A. Beilstein, and

- Rebecca A. Mosher. 2015. "Ancient Origin and Recent Innovations of RNA Polymerase IV and V." *Molecular Biology and Evolution* 32 (7): 1788–99.
- Jeon, J. S., S. Lee, K. H. Jung, S. H. Jun, D. H. Jeong, J. Lee, C. Kim, et al. 2000. "T-DNA Insertional Mutagenesis for Functional Genomics in Rice." *The Plant Journal: For Cell and Molecular Biology* 22 (6): 561–70.
- Jeong, Dong-Hoon, Suyoung An, Sunhee Park, Hong-Gyu Kang, Gi-Gyeong Park, Sung-Ryul Kim, Jayeon Sim, et al. 2006. "Generation of a Flanking Sequence-Tag Database for Activation-Tagging Lines in Japonica Rice." *The Plant Journal: For Cell and Molecular Biology* 45 (1): 123–32.
- Jiang, Ning, and Olivier Panaud. 2013. "Transposable Element Dynamics in Rice and Its Wild Relatives." In *Genetics and Genomics of Rice*, 55–69. New York, NY: Springer New York.
- Kalvari, Ioanna, Eric P. Nawrocki, Joanna Argasinska, Natalia Quinones-Olvera, Robert D. Finn, Alex Bateman, and Anton I. Petrov. 2018. "Non-Coding RNA Analysis Using the Rfam Database." *Current Protocols in Bioinformatics / Editorial Board, Andreas D. Baxeavanis ... [et Al.]* 62 (1): e51.
- Kanno, Tatsuo, Bruno Huettel, M. Florian Mette, Werner Aufsatz, Estelle Jaligot, Lucia Daxinger, David P. Kreil, Marjori Matzke, and Antonius J. M. Matzke. 2005. "Atypical RNA Polymerase Subunits Required for RNA-Directed DNA Methylation." *Nature Genetics* 37 (7): 761–65.
- Kasschau, Kristin D., Noah Fahlgren, Elisabeth J. Chapman, Christopher M. Sullivan, Jason S. Cumbie, Scott A. Givan, and James C. Carrington. 2007. "Genome-Wide Profiling and Analysis of Arabidopsis siRNAs." *PLoS Biology* 5 (3): e57.
- Krueger, Felix. v0.6.5 2019. "TrimGalore: A Wrapper around Cutadapt and FastQC to Consistently Apply Adapter and Quality Trimming to FastQ Files, with Extra Functionality for RRBS Data." v0.6.5 2019. <https://github.com/FelixKrueger/TrimGalore>.
- Kyle Littlefield and Matthew Monroe. 2020. "Venn-Diagram-Plotter: The Venn Diagram Plotter Draws Correctly Proportioned and Positioned Two and Three Circle Venn Diagrams (Aka Euler Diagrams)." June 2, 2020. <https://github.com/PNNL-Comp-Mass-Spec/Venn-Diagram-Plotter>.
- Langmead, Ben, Cole Trapnell, Mihai Pop, and Steven L. Salzberg. 2009. "Ultrafast and Memory-Efficient Alignment of Short DNA Sequences to the Human Genome." *Genome Biology* 10 (3): R25.
- Li, Carey Fei, Olga Pontes, Mahmoud El-Shami, Ian R. Henderson, Yana V. Bernatavichute, Simon W-L Chan, Thierry Lagrange, Craig S. Pikaard, and Steven E. Jacobsen. 2006. "An ARGONAUTE4-Containing Nuclear Processing Center Colocalized with Cajal Bodies in Arabidopsis Thaliana." *Cell* 126 (1): 93–106.
- Li, Qing, Jonathan I. Gent, Greg Zynda, Jawon Song, Irina Makarevitch, Cory D. Hirsch, Candice N. Hirsch, et al. 2015. "RNA-Directed DNA Methylation Enforces Boundaries between Heterochromatin and Euchromatin in the Maize Genome." *Proceedings of the National Academy of Sciences of the United States of America* 112 (47): 14728–33.
- Luo, Jie, and Benjamin D. Hall. 2007. "A Multistep Process Gave Rise to RNA Polymerase IV of Land Plants." *Journal of Molecular Evolution* 64 (1): 101–12.

- Mi, Shijun, Tao Cai, Yugang Hu, Yemiao Chen, Emily Hodges, Fangrui Ni, Liang Wu, et al. 2008. "Sorting of Small RNAs into Arabidopsis Argonaute Complexes Is Directed by the 5' Terminal Nucleotide." *Cell* 133 (1): 116–27.
- Moritoh, Satoru, Chang Ho Eun, Akemi Ono, Hisayo Asao, Yosuke Okano, Katsushi Yamaguchi, Zenpei Shimatani, Amame Koizumi, and Rie Terada. 2012. "Targeted Disruption of an Orthologue of DOMAINS REARRANGED METHYLASE 2, OsDRM2, Impairs the Growth of Rice Plants by Abnormal DNA Methylation." *The Plant Journal: For Cell and Molecular Biology* 71 (1): 85–98.
- Mosher, Rebecca A., Frank Schwach, David Studholme, and David C. Baulcombe. 2008. "PolIVb Influences RNA-Directed DNA Methylation Independently of Its Role in SiRNA Biogenesis." *Proceedings of the National Academy of Sciences of the United States of America* 105 (8): 3145–50.
- Onodera, Yasuyuki, Jeremy R. Haag, Thomas Ream, Pedro Costa Nunes, Olga Pontes, and Craig S. Pikaard. 2005. "Plant Nuclear RNA Polymerase IV Mediates SiRNA and DNA Methylation-Dependent Heterochromatin Formation." *Cell* 120 (5): 613–22.
- Pontier, Dominique, Galina Yahubyan, Danielle Vega, Agnès Bulski, Julio Saez-Vasquez, Mohamed-Ali Hakimi, Silva Lerbs-Mache, Vincent Colot, and Thierry Lagrange. 2005. "Reinforcement of Silencing at Transposons and Highly Repeated Sequences Requires the Concerted Action of Two Distinct RNA Polymerases IV in Arabidopsis." *Genes & Development* 19 (17): 2030–40.
- Quinlan, Aaron R., and Ira M. Hall. 2010. "BEDTools: A Flexible Suite of Utilities for Comparing Genomic Features." *Bioinformatics* 26 (6): 841–42.
- Ream, Thomas S., Jeremy R. Haag, Andrzej T. Wierzbicki, Carrie D. Nicora, Angela D. Norbeck, Jian-Kang Zhu, Gretchen Hagen, Thomas J. Guilfoyle, Ljiljana Paša-Tolić, and Craig S. Pikaard. 2009. "Subunit Compositions of the RNA-Silencing Enzymes Pol IV and Pol V Reveal Their Origins as Specialized Forms of RNA Polymerase II." *Molecular Cell* 33 (2): 192–203.
- Sidorenko, Lyudmila, Jane E. Dorweiler, A. Mark Cigan, Mario Arteaga-Vazquez, Meenal Vyas, Jerry Kermicle, Diane Jurcin, Jan Brzeski, Yu Cai, and Vicki L. Chandler. 2009. "A Dominant Mutation in Mediator of Paramutation2, One of Three Second-Largest Subunits of a Plant-Specific RNA Polymerase, Disrupts Multiple SiRNA Silencing Processes." *PLoS Genetics* 5 (11): e1000725.
- Singh, Jasleen, Vibhor Mishra, Feng Wang, Hsiao Yun Huang, and Craig S. Pikaard. 2019. "Reaction Mechanisms of Pol IV, RDR2, and DCL3 Drive RNA Channeling in the SiRNA-Directed DNA Methylation Pathway." *Molecular Cell* 75 (3): 576-589.e5.
- Song, Xianwei, and Xiaofeng Cao. 2017. "Transposon-Mediated Epigenetic Regulation Contributes to Phenotypic Diversity and Environmental Adaptation in Rice." *Current Opinion in Plant Biology* 36 (April): 111–18.
- Stonaker, Jennifer L., Jana P. Lim, Karl F. Erhard Jr, and Jay B. Hollick. 2009. "Diversity of Pol IV Function Is Defined by Mutations at the Maize Rmr7 Locus." *PLoS Genetics* 5 (11): e1000706.
- Stroud, Hume, Truman Do, Jiamu Du, Xuehua Zhong, Suhua Feng, Lianna Johnson, Dinshaw J. Patel, and Steven E. Jacobsen. 2014. "Non-CG Methylation Patterns Shape the

- Epigenetic Landscape in Arabidopsis." *Nature Structural & Molecular Biology* 21 (1): 64–72.
- Stroud, Hume, Maxim V. C. Greenberg, Suhua Feng, Yana V. Bernatavichute, and Steven E. Jacobsen. 2013. "Comprehensive Analysis of Silencing Mutants Reveals Complex Regulation of the Arabidopsis Methylome." *Cell* 152 (1): 352–64.
- Trujillo, Joshua T., Arun S. Seetharam, Matthew B. Hufford, Mark A. Beilstein, and Rebecca A. Mosher. 2018. "Evidence for a Unique DNA-Dependent RNA Polymerase in Cereal Crops." *Molecular Biology and Evolution* 35 (10): 2454–62.
- Tucker, S. L., J. Reece, T. S. Ream, and C. S. Pikaard. 2010. "Evolutionary History of Plant Multisubunit RNA Polymerases IV and V: Subunit Origins via Genome-Wide and Segmental Gene Duplications, Retrotransposition, and Lineage-Specific Subfunctionalization." *Cold Spring Harbor Symposia on Quantitative Biology* 75: 285–97.
- Wang, Yaqiong, and Hong Ma. 2015. "Step-Wise and Lineage-Specific Diversification of Plant RNA Polymerase Genes and Origin of the Largest Plant-Specific Subunits." *The New Phytologist* 207 (4): 1198–1212.
- Wang, Zhenxing, Nicolas Butel, Juan Santos-González, Filipe Borges, Jun Yi, Robert A. Martienssen, German Martinez, and Claudia Köhler. 2020. "Polymerase IV Plays a Crucial Role in Pollen Development in Capsella." *The Plant Cell* 32 (4): 950–66.
- Wei, Liya, Lianfeng Gu, Xianwei Song, Xiekui Cui, Zhike Lu, Ming Zhou, Lulu Wang, et al. 2014. "Dicer-like 3 Produces Transposable Element-Associated 24-Nt siRNAs That Control Agricultural Traits in Rice." *Proceedings of the National Academy of Sciences of the United States of America* 111 (10): 3877–82.
- White, J. L., and J. M. Kaper. 1989. "A Simple Method for Detection of Viral Satellite RNAs in Small Plant Tissue Samples." *Journal of Virological Methods* 23 (2): 83–93.
- Wierzbicki, Andrzej T., Jeremy R. Haag, and Craig S. Pikaard. 2008. "Noncoding Transcription by RNA Polymerase Pol IVb/Pol V Mediates Transcriptional Silencing of Overlapping and Adjacent Genes." *Cell* 135 (4): 635–48.
- Wierzbicki, Andrzej T., Thomas S. Ream, Jeremy R. Haag, and Craig S. Pikaard. 2009. "RNA Polymerase v Transcription Guides ARGONAUTE4 to Chromatin." *Nature Genetics* 41 (5): 630–34.
- Xie, Zhixin, Lisa K. Johansen, Adam M. Gustafson, Kristin D. Kasschau, Andrew D. Lellis, Daniel Zilberman, Steven E. Jacobsen, and James C. Carrington. 2004. "Genetic and Functional Diversification of Small RNA Pathways in Plants." *PLoS Biology* 2 (5): E104.
- Xu, Le, Kun Yuan, Meng Yuan, Xiangbing Meng, Min Chen, Jianguo Wu, Jiayang Li, and Yijun Qi. 2020. "Regulation of Rice Tillering by RNA-Directed DNA Methylation at Miniature Inverted-Repeat Transposable Elements." *Molecular Plant* 13 (6): 851–63.
- Zemach, Assaf, M. Yvonne Kim, Ping Hung Hsieh, Devin Coleman-Derr, Leor Eshed-Williams, Ka Thao, Stacey L. Harmer, and Daniel Zilberman. 2013. "The Arabidopsis Nucleosome Remodeler DDM1 Allows DNA Methyltransferases to Access H1-Containing Heterochromatin." *Cell* 153 (1): 193–205.
- Zhai, Jixian, Sylvain Bischof, Haifeng Wang, Suhua Feng, Tzuu Fen Lee, Chong Teng, Xinyuan Chen, et al. 2015. "A One Precursor One siRNA Model for Pol IV-Dependent siRNA Biogenesis." *Cell* 163 (2): 445–55.

- Zhang, Chao, Ying Wei, Le Xu, Kang-Cheng Wu, Liang Yang, Chao-Nan Shi, Guo-Yi Yang, et al. 2020. "A Bunyavirus-Inducible Ubiquitin Ligase Targets RNA Polymerase IV for Degradation during Viral Pathogenesis in Rice." *Molecular Plant* 13 (6): 836–50.
- Zhang, Huiming, Kai Tang, Bangshing Wang, Cheng-Guo Duan, Zhaobo Lang, and Jian-Kang Zhu. 2014. "Protocol: A Beginner's Guide to the Analysis of RNA-Directed DNA Methylation in Plants." *Plant Methods* 10 (June): 18.
- Zhang, Xiaoyu, Ian R. Henderson, Cheng Lu, Pamela J. Green, and Steven E. Jacobsen. 2007. "Role of RNA Polymerase IV in Plant Small RNA Metabolism." *Proceedings of the National Academy of Sciences of the United States of America* 104 (11): 4536–41.
- Zheng, Kezhi, Lili Wang, Longjun Zeng, Dachao Xu, Zhongxin Guo, Xiquan Gao, and Dong-Lei Yang. 2021. "The Effect of RNA Polymerase V on 24-Nt siRNA Accumulation Depends on DNA Methylation Contexts and Histone Modifications in Rice." *Proceedings of the National Academy of Sciences of the United States of America* 118 (30). <https://doi.org/10.1073/pnas.2100709118>.
- Zhong, Xuehua, Christopher J. Hale, Julie A. Law, Lianna M. Johnson, Suhua Feng, Andy Tu, and Steven E. Jacobsen. 2012. "DDR Complex Facilitates Global Association of RNA Polymerase v to Promoters and Evolutionarily Young Transposons." *Nature Structural & Molecular Biology* 19 (9): 870–75.