AN ABSTRACT OF THE THESIS OF

Nicholas Lowery for the degrees of Honors Baccalaureate of Science in Microbiology and Honors Baccalaureate of Science in Biochemistry and Biophysics presented July 12, 2011. Title: The Role of *Arabidopsis* RNA-Dependent RNA Polymerase Genes 3, 4 and 5 in Antiviral Defense.

Abstract Approved: _____
James C. Carrington

RNA silencing plays a critical role in plant defense against viral infection. Plants use virus-derived small interfering RNA to target and silence invading viruses. The antiviral silencing pathway can be broken down into three conceptual stages: initiation, amplification, and systemic movement. The molecular mechanisms of silencing initiation are not well understood, but may involve dicer-like endonucleases and/or Argonaute proteins in complex with small RNAs. Silencing amplification, on the other hand, is mediated by RNA-dependent RNA polymerases. In the model species *Arabidopsis thaliana*, there are six known RNA-dependent RNA polymerase (RDR) genes. Three of these genes (RDR1, RDR2 and RDR6) have been biologically characterized, and found to be active during the antiviral response. However, no function has been assigned to RDR3, RDR4 or RDR5. In this study, we sought to investigate whether RDR3, RDR4 or RDR5 genes participated in antiviral defense using *A. thaliana* and *Turnip mosaic virus*. Results obtained from single and quadruple mutant lines indicate that RDR3, RDR4 and RDR5 do not function in the main antiviral defense pathway in *A. thaliana*. Instead, the activity, if any, would be secondary to the pathways mediated by RDR1 and RDR6, and likely redundant between RDR3, RDR4 and RDR5.

Key words: RDR, *A. thaliana*, antiviral silencing, TuMV

Corresponding email address: loweryni@onid.orst.edu

The Role of *Arabidopsis* RNA-Dependent RNA Polymerase Genes 3, 4 and 5 in Antiviral Defense

By

Nicholas Lowery

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APPROVED:

_____

Mentor, representing Botany and Plant Pathology

_____

Committee Member, representing Botany and Plant Pathology

_____

Committee Member, representing Microbiology

_____

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Nicholas Lowery, Author

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The Role of *Arabidopsis* RNA-Dependent RNA Polymerase Genes 3, 4 and 5 in Antiviral Defense

INTRODUCTION

Viruses infect all known organisms, from bacteria to plants to mammals, and infections in each clade produce unique and significant effects. For instance, plant viruses greatly reduce yields from many agriculturally important species. Thus, understanding how organisms react to viral infection at the cellular level is of the utmost importance in both prevention of outbreaks and remediation of existing problems. Plants employ a variety of mechanisms to defend themselves from pathogens at both molecular and organismal levels, including innate immunity (9) and RNA silencing (5).

Antiviral RNA silencing is a critical mechanism of viral resistance in plants, inhibiting viral replication and spread at both the cellular and systemic levels (10). The pathway relies on various members of several key enzyme families, reviewed in (2, 5). Briefly, antiviral RNA silencing is triggered by the presence of double stranded RNA (dsRNA), which is processed into 21-24 nucleotide fragments (called primary small interfering RNA or siRNA) by a Dicer-like ribonuclease (DCL). The siRNA are then bound by a member of the ARGONAUTE (AGO) proteins to form the active center of an RNA-induced silencing complex (RISC). The RISC will then selectively bind RNAs containing sequences complementary to the incorporated siRNA. In the context of virus-derived siRNAs, RISCs will target the viral genomic or messenger RNAs. Once bound to the target, the RISC can then enzymatically cleave the target RNA, which is then degraded, or remain bound and function as a translational inhibitor.

Thus, by binding or cleaving target viral RNAs, RISC functions as a major antiviral effector at the cellular level. However, through the actions of an RNA-Dependent RNA Polymerase (RDR), the plant can amplify the antiviral response leading to a widespread antiviral state and systemic immunity to viral infection (2, 5). When the RISC cleaves the target RNA, an RDR can bind to the cleavage fragments and generate a new dsRNA molecule. This new dsRNA can then re-enter the pathway, generating secondary siRNAs also capable of silencing the virus. Successive rounds of this RDR-dependent amplification pathway results in large numbers of secondary siRNAs, which, in addition to acting locally, can spread throughout the plant over short distances via plasmodesmata and systemically through the vasculature (2, 5).

Six RDR genes have been identified in *Arabidopsis thaliana*, three of which have been characterized. RDR1 has been associated primarily with antiviral defense mechanisms (6, 8), while RDR2 plays a role in DNA methylation (3), and RDR6 functions in both endogenous trans-acting siRNA biogenesis as well as antiviral silencing (4, 6, 11). In contrast, biological functions for RDR3, RDR4 and RDR5 remain to be elucidated.

Garcia-Ruiz et al. (6) noted that siRNA biogenesis during infection by *Turnip mosaic virus* (TuMV) was greatly reduced, but not completely eliminated, in *rdr1-1 rdr2-1 rdr6-15* triple mutant *A. thaliana* plants. The source of these basal virus-derived siRNAs is currently unknown. In this study, we tested the hypothesis that RDR3, RDR4 or RDR5 in *A. thaliana* participate in the biogenesis of virus-derived siRNA.

MATERIALS AND METHODS

Plant Materials

A. thaliana Col-0, *dcl2-1 dcl3-1 dcl4-2* and *rdr1-1 rdr2-1 rdr6-15* lines have been described previously (6). Null allele *rdr3-2*, *rdr4-2* and *rdr5-3* single mutant lines were obtained from the Salk Institute (1). Quadruple mutant lines (*rdr1-1 rdr2-1 rdr3-2 rdr6-15, rdr1-1 rdr2-1 rdr4-2 rdr6-15* and *rdr1-1 rdr2-1 rdr5-3 rdr6-15*) were made by conventional crossing. Plants were maintained at 22°C in 16 hr light / 8 hr dark cycles in a growth room. Three-primer PCR reactions were used to genotype all lines.

Virus Propagation and Inoculum Preparation

TuMV-GFP was propagated in *Nicotiana benthamiana* as described (7).

Infection Assays

Infection assays were carried out as described (6). Briefly, 3 microliters (μL) of inoculum were rub-incoluated into four carborundum-dusted leaves of 30 day old *A. thaliana* plants, eight per genotype. Control plants were mock-inoculated with phosphate buffer. Infection efficiency and virus accumulation were determined by counting GFP-fluorescent foci on infected leaves and western blotting, respectively. Total protein was extracted from pooled inflorescence clusters collected at 15 dpi. 6.25 micrograms (μg) of total protein was used for blotting. TuMV-CP was detected using PVAS-134 antibody (1:5000 dilution) and Western Lightning plus-ECL substrate (Perkin-Elmer). Inflorescence clusters were concomitantly collected at 15 dpi for total RNA extraction. 15 μg of total RNA was subjected to northern blotting and probed for the CI region (sense and antisense) of TuMV. Hybridization intensities were normalized to U6 RNA.

RESULTS

Virus Infection Efficiency

If RDR3, RDR4 and RDR5 play a role in the biogenesis of TuMV-derived siRNAs, we predict that in the absence of these genes, virus-derived siRNAs will accumulate to lower levels compared to wild type plants. To test this hypothesis, we screened both single and combination *rdr3-2*, *rdr4-2* and *rdr5-3* mutant *A. thaliana* for changes in infection efficiency and levels of virus-derived siRNAs as compared to *rdr1-1 rdr 2-1 rdr6-15* triple mutant plants. No change was detected in infection efficiency of inoculated leaves for *rdr3-2*, *rdr4-2* or *rdr5-3* single mutant plants or in combination with *rdr1-1 rdr2-1* and *rdr6-15* (Figure 1A). Similar results were observed in inflorescence tissue (Figure 1B). Thus, the lack of RDR3, RDR4 or RDR5 had no effect on virus infection.

Accumulation of Virus-Derived siRNAs

rdr3-2, rdr4-2 or *rdr5-3* mutant plants accumulated virus-derived siRNAs to levels similar to wild type plants, while the quadruple mutants accumulated similar levels to the *rdr1-1 rdr2-1 rdr6-15* triple mutants (Figure 2A). Additionally, no significant difference was detected between sense and antisense siRNAs in any of the mutant lines (Figure 2B). These results indicate that RDR3, RDR4 and RDR5 do not take part in the antiviral silencing pathway in *A. thaliana*. Alternatively, any effects on the biogenesis of TuMV-derived siRNAs provided by RDR3, RDR4 or RDR5 may be redundant between these genes.

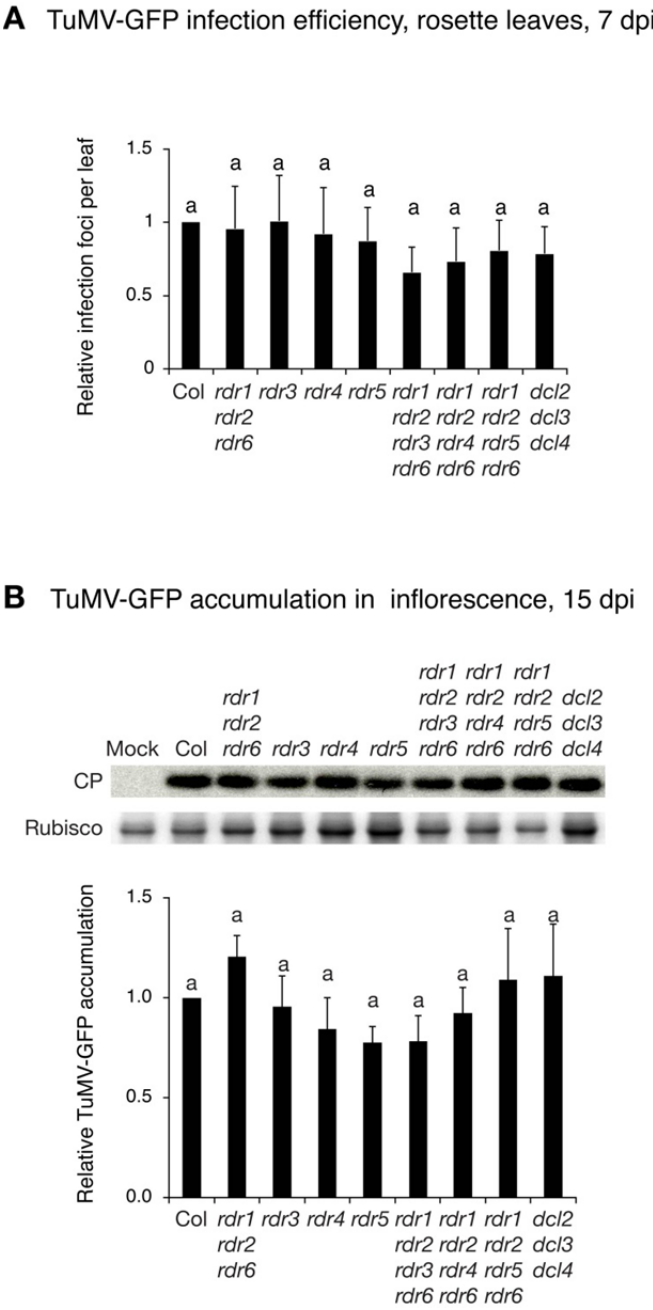

Figure 1: Local and systemic infection of *A. thaliana rdr* mutants by TuMV-GFP. A) Normalized GFP foci in infected rosette leaves. Average foci counts (+ SE) are plotted relative to Col-0. B) TuMV accumulation in inflorescence tissue. Blots display representative results from westerns. Average CP + SE is plotted relative to Col-0. Letters in A) and B) denote no significant difference in infection levels was detected (Tukey's test with $\alpha = 0.01$).

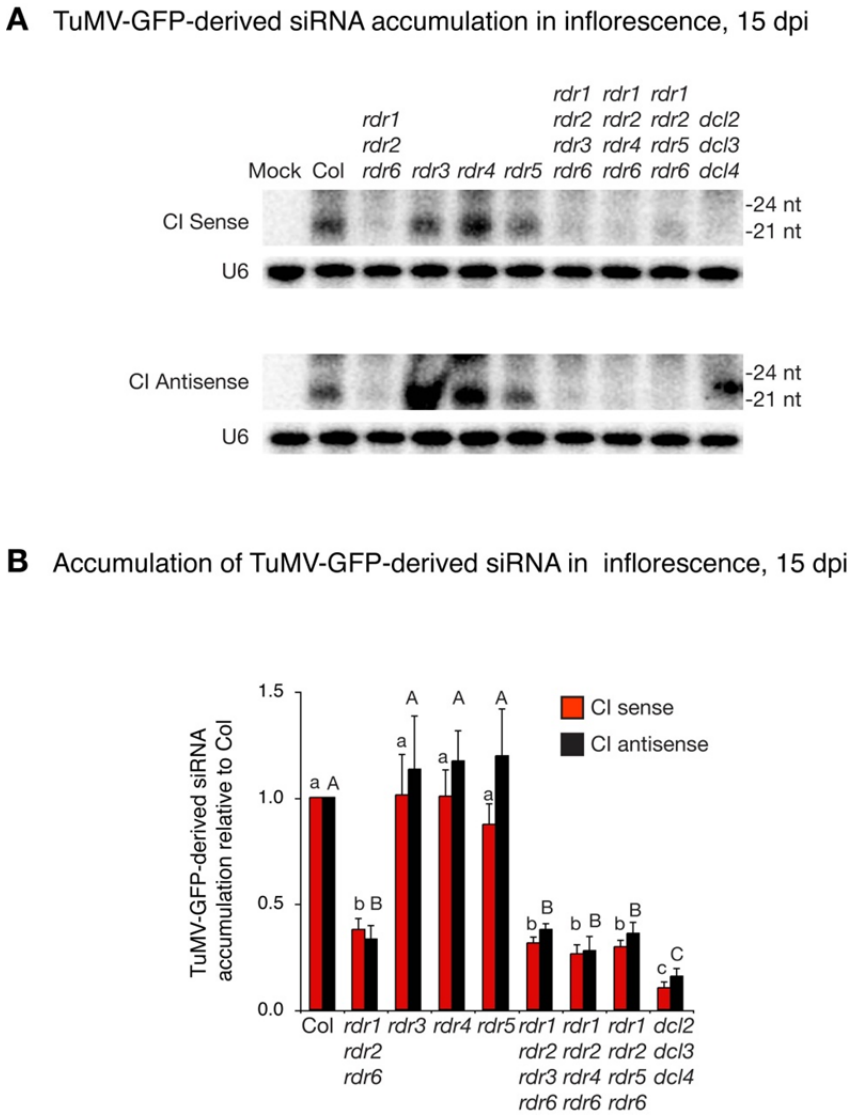

Figure 2: siRNA accumulation in *A. thaliana* inflorescence showing systemic TuMV-GFP infection. A) Representative northern blots. Total RNA was probed for TuMV-derived siRNA in both the sense and antisense directions. B) Histogram summarizing results of northern blots. Average intensities are plotted (+SE) relative to Col-0. Letters designate significance groups for average intensities (Tukey's test with $\alpha = 0.01$), lower-case indicates sense strand siRNAs, upper-case denotes antisense siRNAs.

DISCUSSION

The results of this study support a model in which *A. thaliana* genes RDR3, RDR4 and RDR5 do not participate in antiviral RNA silencing against TuMV, or their activities are redundant to each other and secondary to the pathways dependent on RDR1 and RDR6. Using the quadruple mutants described here, we cannot rule out the possibility of redundancy; however, this could be studied by simultaneously down-regulating RDR3, RDR4 and RDR5 using artificial microRNAs in Col-0 and *rdr1 rdr2 rdr6* triple mutants, thereby creating *rdr3 rdr4 rdr5* triple mutant or *rdr-null* (*rdr1-1 rdr2-1 rdr3-2 rdr4-2 rdr5-3 rdr6-15* sextuple mutant) *A. thaliana* lines.

The source of virus-derived siRNAs generated in *rdr1 rdr2 rdr6* triple mutants remains to be determined. An interesting alternative hypothesis is that those virus-derived siRNAs are RDR-independent primary siRNAs generated by Dicer-like endonucleases in the initial steps of infection. Clear distinction between primary and secondary virus-derived siRNAs has not been reported. However, the quadruple mutant plants from this study could be used to map the source of RDR-independent virus-derived siRNAs. This experiment would require construction of small RNA libraries from the *rdr* quadruple mutants reported here, as well as an *rdr-null* line. Sequencing the siRNAs generated by TuMV-infected *rdr-null* plants and comparison to the siRNA profile to *rdr1-1 rdr2-1 rdr6-15* triple mutants could then identify primary siRNAs. Such an experiment may provide valuable insight into the nature of sources for primary siRNA biogenesis across the TuMV genome.

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