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Exploration of Antiviral Effects of RNA-dependent RNA Polymerases 3, 4 and 5 in *Arabidopsis*

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Abstract

Small RNAs play a number of vital roles in plants, including viral resistance. A particular class of small RNA that regulates plant defense from pathogens, among other functions, is known as small interfering RNAs (siRNAs). A key player in the biogenesis pathway of siRNAs are RNA-Dependent RNA Polymerases (RDRs). There are six RDR proteins in Arabidopsis thaliana, three of which have well documented roles (RDRs 1, 2 and 6), and three of which have no documented role. Here, we look at the functions of the unstudied RDRs, RDRs 3, 4 and 5, to see whether they affect antiviral resistance.

Keywords: small RNA, siRNA, RNA dependent RNA polymerases, plant biology, molecular biology, molecular genetics, plant pathology

Introduction

The biggest challenge faced in modern agriculture is increasing food production to meet the demands of a growing population. Disease plays a major role in crop failure, with losses to staple crops due to pests and disease ranging between 10-40% [1], [2]. This effect is being compounded by plants' increased susceptibility to disease in the face of global climate change [3]. As such, understanding the factors that increase plant resistance to disease is crucial to our efforts towards sustainable agriculture. One of the key viral resistance mechanisms in plants is RNA-mediated silencing. This mechanism is carried out by small RNAs (sRNA), noncoding signaling molecules that range between 21 and 24 nucleotides in length. Small RNAs play essential roles in plant development, reproduction and defense [4]. In particular, those that regulate defense responses in plants belong to a class known as small interfering RNAs (siRNAs) [5].

These siRNAs are produced from a double-stranded RNA (dsRNA) precursor, which is cleaved by a DICER-LIKE (DCL) protein. siRNAs then form a complex with ARGONAUTE

(AGO) proteins and target complementary nucleotide sequences, effectively silencing the expression of the target gene [6]. A major step in siRNA biogenesis, the precursor synthesis, is what differentiates siRNAs from other sRNAs. The dsRNA precursor is a product of RNA-dependent RNA polymerase (RDR), which uses single-stranded RNA (ssRNA) as a template to generate the second strand [5].

RDR is an ancient protein family that is intrinsically linked with the evolution of RNA viruses and plays essential roles in their RNA replication [7]. RDRs have been conserved throughout the evolution of plants, functioning to synthesize dsRNA from ssRNA [6]. There are 6 different types of RDRs found in *Arabidopsis thaliana* [8]. RDRs 1, 2 and 6 are well studied, and have been shown to generate siRNAs involved in mediating plant stress response, pathogen resistance, female gamete formation, transgene silencing, and plant development [9]. However, the roles of RDRs 3, 4 and 5 and their sRNA products have not been well explored to date [8].

Being so highly conserved across kingdoms, and because of the vital roles that RDRs 1, 2 and 6 play, we believe that studying the functions of RDRs 3, 4 and 5 is essential. Two major lines of evidence indicate that RDR-derived sRNA silencing forms the core of an antiviral defense in plants: (1) siRNAs targeting viral RNA accumulate during infections; and (2) viruses produce virulence factors called viral suppressors of RNA silencing to counteract this defense [10]. This raises a question as to what role, if any, do RDRs 3, 4 and 5 play in antiviral defense.

Materials and Methods

Plant Materials

We used nine different *Arabidopsis thaliana* genotypes, including wildtype *Columbia-0 (Col-0)* and the following mutants: *rdr1-1*, *rdr2-1*, *rdr3-2*, *rdr4-2*, *rdr5-3*, *rdr6-4*, *ago2-1*, and *dcl2/3/4* [11]. All the mutant lines were received from the Carrington lab at the Donald Danforth Plant Science Center and are in *Col-0* background. Plants were grown in growth chambers under long day conditions (16-hour light/8-hour dark) at 21°C and 50% relative humidity. The *rdr3, 4* and *5* mutants used in this study are SALK lines (Accession numbers: Salk 036925, Salk 088175, Salk 023522).

Nicotiana benthamiana plants were used to establish viral inoculum used for *Arabidopsis* infection. *Nicotiana* plants were grown in greenhouses at 28°C (day temperature) and 25°C (night temperature) at 60% relative humidity. Watering was stopped two days pre infiltration to increase leaf permeability.

DNA Plasmids

DNA plasmids containing CRISPR constructs were generated to create the following knockout lines: *rdr3, rdr4, rdr5, rdr3/4, rdr3/5, rdr4/5 and rdr3/4/5.* Guide RNA constructs were assembled using the Goldengate method. The constructs were inserted into the destination vector containing a Cas9 coding sequence via Gateway LR reaction. The inserts were confirmed by sequencing and final plasmids were transformed into *Agrobacterium tumefaciens* LBA4404.

TCV Virus Infection Assays

Plasmids carrying TCV, TCV-CPB and P19 were shared by Dr. Zheng [12]. Inoculum preparation and inoculation of *Arabidopsis thaliana* were performed as described [12]. Briefly, *Nicotiana benthamiana* leaves were infiltrated with each of the infection clones. Infected leaves were collected 5 days after infiltration (DAI) and ground in 200mM NaOAc. After overnight incubation at 4°C in a PEG-8000 and NaCl solution, virions were pelleted by centrifugation and resuspended in 10mM NaOAc. This inoculum stock was diluted 10 times with 10mM NaOAc and used to inoculate the 4 largest *Arabidopsis* rosette leaves.

TuMV Virus Infection Assay

Plasmids carrying TuMV-GFP were shared by the Carrington lab [13]. *Nicotiana benthamiana* plants

were infiltrated as described previously for inoculum preparation [14]. Infection development was confirmed by GFP fluorescence under UV light at 6DAI. Infected leaves were collected and inoculum prepared as described [13].

Image Capture

Images for phenotyping were captured by Raspberry Pi cameras mounted directly over the plants in the growth chamber. Plants of different treatment and genotypes were placed randomly throughout the chamber and assigned a code for identification purposes. The cameras were programmed to capture one image every hour starting from 2 days pre-infection to 14 days post-infection. One camera was used per tray. Black mesh was placed around the plants to eliminate background interference.

Image Analysis

Images taken every day at 11am were analyzed using PlantCV, a python-based phenotyping program. PlantCV was used to identify and segment plant leaves and distinguish them from the background mesh. Spots of necrosis (brown) and chlorosis (yellowing) were identified against healthy leaf tissue (green). Pixel counts for healthy and unhealthy tissue were collected for each plant.

Statistical Analysis

All statistical analysis was performed in R. Proportion of necrotic tissue per day was calculated for each plant. Averages of necrotic tissue proportion were taken per genotype for each treatment each day. Kolmogorov-Smirnov tests were run for each genotype compared to Col-0 wt to test whether the distribution curves were significantly different from one another.

Results

Image-Based Analysis of Disease Symptoms

Plants that were infected by TuMV and TCV showed more severe symptoms than those infected with TCV-CPB. Overall, we found no evidence to suggest that RDRs 3, 4 or 5 play any role in antiviral defense in *Arabidopsis*.

When graphed, our positive control mutant, *dcl2/3/4*, showed the greatest necrotic tissue expansion across all viral treatments, while our positive control, wt Col0, showed the least necrotic tissue expansion across almost all treatments (see Figure 1).

We used the Kolmogorov-Smirnov (K-S) test to determine whether or not the distributions for each genotype were different than our Col0 control. The output from the K-S test is a p-value that states whether or not two series of data were drawn from the same distribution. For most of our genotypes across treatments, p-values did not meet our required significance level of 0.05 (see Table 1). However, we did see that *rdr5* mutants had a significantly different distribution curve than Col0 after TCV infection (p-value: .0015). We also saw that *rdr4* mutants had significantly different distributions than Col0 after Mock and TCV infections, though necrotic tissue progression continued more slowly than Col0 in the case of TCV.

RDR4 Mutants Exhibit Significant Developmental Delay

rdr4 mutants consistently displayed stunted growth when compared to the other mutants that we observed. Plants were significantly smaller than wild-type Col0 of the same age (p-value: 3.57×10^{-7}, see Figure 2).

Discussion

Although we completed only one full replicate, we found no significant evidence that RDRs 3, 4 and 5 function in viral defense in plants. We think that the significant p-values for *rdr4* mutants in Table 1 are due to inconsistencies in their growth rate and that their stunted growth doesn't allow for accurate analysis of proportion of necrotic tissue in the plant. We attribute this to there being insufficient total tissue area for the calculation to be meaningful without more replicates. Although *rdr5* mutants exhibited a significant p-value compared to Col0 after TCV infection, the plants started out with more

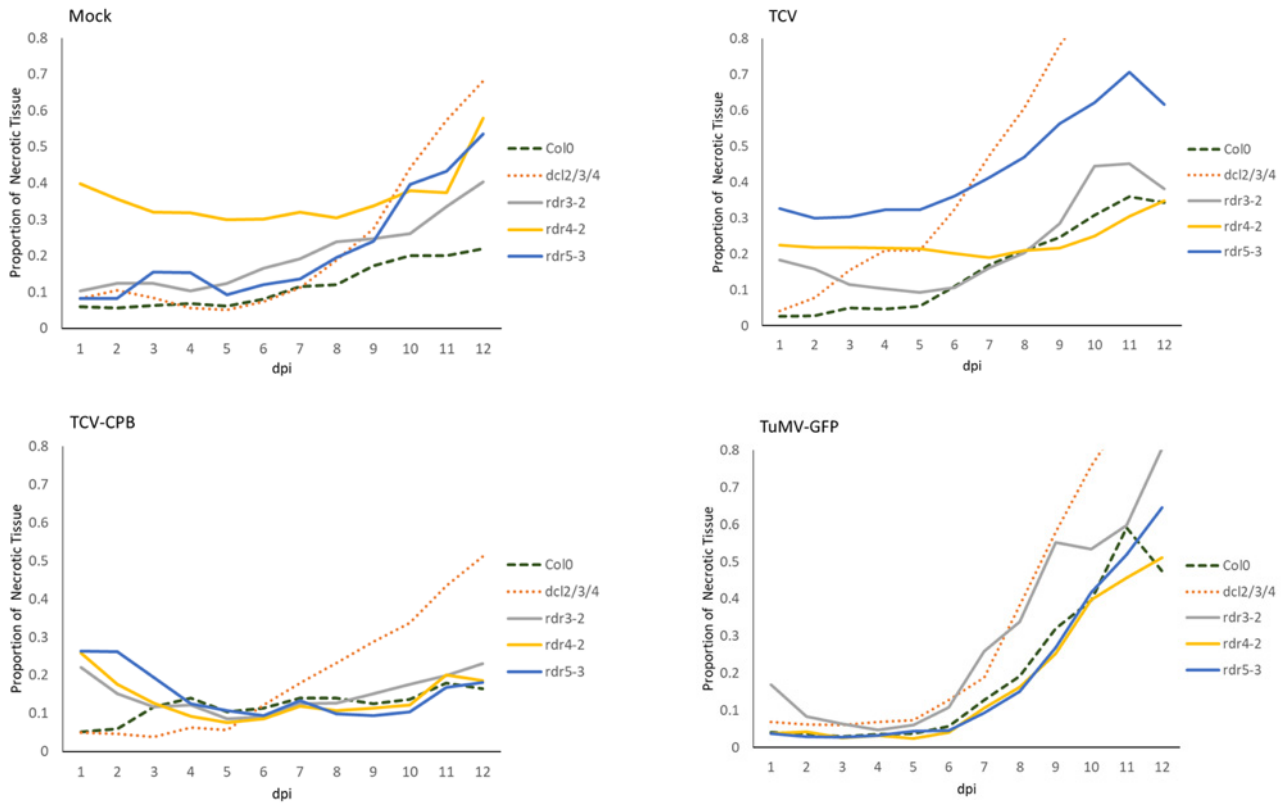

Figure 1. Line graphs showing the progression of necrotic tissue expansion over time after infection of *Arabidopsis* with Mock, TCV, TCV-CPB, and TuMV-GFP viruses.

Table 1. P-values from K-S test for each genotype against Col0 wildtype, separated by treatment.

Genotype	Mock p-values	TCV p-values	TuMV p-values	TCV-CPB p-values
dcl2/3/4	0.54	0.10	0.10	0.10
rdr3	0.10	0.26	0.26	0.54
rdr4	7.40E-07	0.03	1.00	0.87
rdr5	0.10	1.50E-03	1.00	0.54

necrotic tissue than Col0 and increased at almost the exact same rate as infection progressed. This significant difference, then, is not due to rate of necrotic tissue accumulation.

Because the virus-infected single mutant rdr knockout lines showed no sign of increased susceptibility to viruses, we can conclude that there must be alternative pathways for viral resistance in plants. With evidence to support alternative viral resistance pathways, we propose three likely hypotheses: (1) RDRs function redundantly and other RDRs are able to compensate for the lack of RDR3, 4 or 5; (2) antimicrobial compounds, secondary metabolites and other chemical compounds sufficiently defend plants from viruses; and (3) RDRs 3, 4 and 5 don't function in antiviral defense but do have other functions.

Hypothesis 1: RDRs function redundantly.

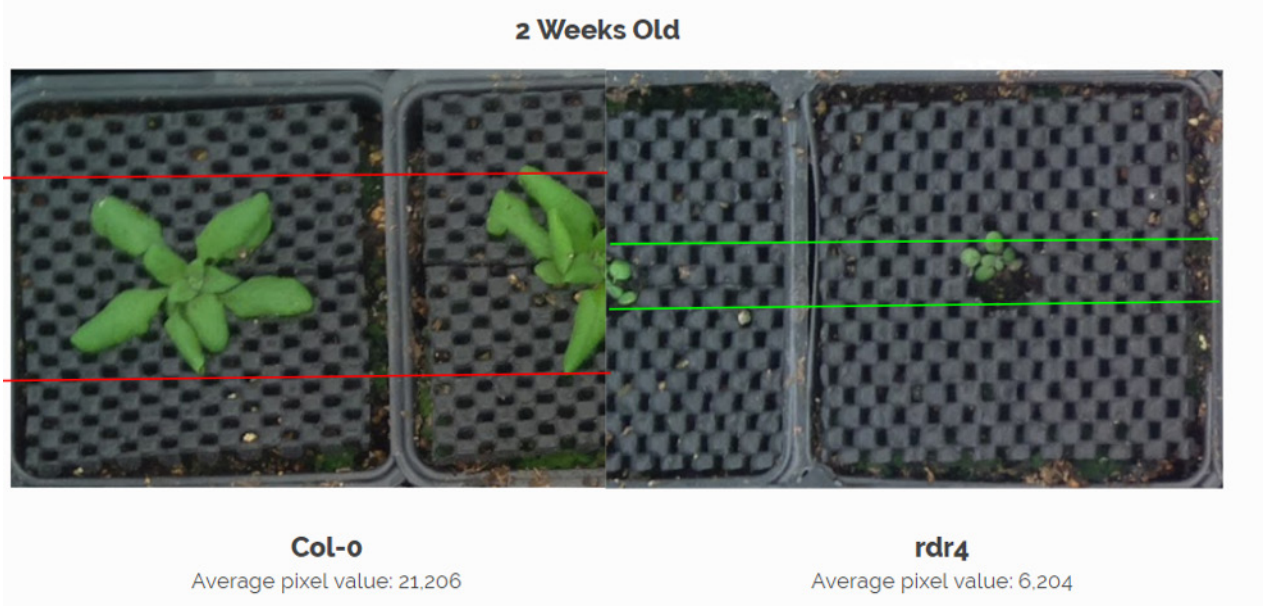

Figure 2. Comparison of 2 week old Col0 wildtype, the day before infection, grown in the same conditions.

There is evidence to support the idea that RDRs function redundantly. RDR1 and RDR6 have both been shown to play a role in antiviral defense [9]. Further, RDRs 3, 4 and 5 are tandem repeats in the *Arabidopsis* genome, suggesting that they arose from a duplication event somewhere along their evolutionary pathway [15]. Because of this potential duplication event, it stands to reason that they might function in the same way. Sequencing the transcriptome of single mutant knockouts could show whether other RDR transcripts were upregulated in the absence of the single RDR that was knocked out. If the RDRs do indeed function redundantly, then it would require multiple knockout mutants (double, triple and even hextuple) to see any effect. We plan to create these mutants and repeat the experiments with the multiple knockout lines.

Hypothesis 2: Antimicrobial compounds, secondary metabolites, and other chemical compounds sufficiently defend plants from viruses. We know that secondary metabolites and antimicrobial compounds protect plants from herbivores and other dangers, so it is likely that they also play a role in viral defense [16].

Hypothesis 3: RDRs 3, 4 and 5 don't function in antiviral defense but do have other functions. From our initial testing, *rdr4* mutants seem to show signs of stunted growth and development. It has also been found that RDR 6 plays a role in eukaryotic cell development, so this would be a natural line of inquiry [9]. It is also possible that RDRs 3, 4 and 5 each have one or many additional roles that we are currently unaware of.

Our most striking result was the discrepancy in growth rate and size between *rdr4* mutant and wildtype Col0. As a result of these findings, we suspect that RDR4 plays a role in growth and development of *Arabidopsis* because of the statistically significant size differences. On average, the *rdr4* mutant was three and a half times smaller than wt at two weeks old before viral infection. We hypothesize that there are transcripts that regulate growth that are targeted by RDR4's siRNA product. Exploring this, possibly by generating and comparing RNA-seq data between the various RDR mutants, seems like a fruitful avenue for further research. Continued research relating to RDR4's role in growth and development, and on the antiviral effects of RDRs 3, 4 and 5, will continue to be important in agriculture and horticulture. The more we understand about disease resistance during the growth and development of plants, the more efficiently we

can engineer our agricultural systems to feed our growing population. Beyond the practical benefits to agriculture, research on the RDRs in *Arabidopsis* will provide further insight into the fundamental molecular processes of their small RNA products. This insight can be leveraged by other scientists to enable deeper research that may provide additional scientific breakthroughs and practical applications.

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