Characterization of RNS2, an S-Like RNase in *Arabidopsis thaliana* suggests role in phosphate recycling

by

Saralyn Ohanian

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Program of Study Committee
Gustavo MacIntosh, Major Professor
Diane Bassham
Jo Anne Powell-Coffman

Iowa State University

Ames, Iowa

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ABSTRACT

Ribonucleases play important biological roles within all organisms but many have not been well characterized. The family of T2 RNases is well-conserved throughout the kingdoms of life and yet their properties and functions are not well understood. *RNS2* is one of five such RNases found in *Arabidopsis*, and preliminary evidence shows that it may be involved in phosphate recycling and localized to an intracellular compartment. Using *rns2* knock-out mutant plants, I showed that RNS2 has an effect on flowering time during times of phosphate starvation. I also showed that RNS2 is localized to the ER, more specifically, to ER-bodies. To explore the hypothesis that a homologous gene from *C.elegans* (*CeRNS*) plays a role in RNAi in nematodes I used RNAi to reduce CeRNS expression, but I did not observe obvious phenotype abnormalities. Additionally, I did not observe consistent effects of CeRNS RNAi on *C. elegans*' response to RNAi.

CHAPTER I: GENERAL INTRODUCTION

Ribonucleases are enzymes that hydrolyze ribonucleic acid (RNA) molecules into individual ribonucleotides or into smaller RNA molecules. They are found in prokaryotes and eukaryotes, and collectively can have diverse roles within cells. An RNase can be classified into many different categories depending upon its characteristics, such as specificity, function, structure, and/or localization (secreted or non-secreted). Because RNases catalyze important reactions, their functions must become understood to further our knowledge of how organisms function.

One super-family of RNases is known as the T2 RNases, all of which belong to the secretory pathway and are targeted to the ER, vacuoles, and extracellular space. T2 RNases are found to be highly conserved throughout the kingdoms of life. Their presence in almost all organisms so far analyzed suggest that they perform an important function that has been maintained (or selected) throughout evolution. Preliminary experiments have hinted at possible T2 functions that include: phosphate recycling which may be part of nutritional and senescing processes, self-incompatibility in plants, and defense against pathogens [16].

One of the most studied groups of T2 RNases is known as the S-RNases, named for their involvement in self-incompatibility in the Rosaceae, Scrophulariaceae, and Solanaceae families of plants [35]. Using conserved sequences within S-RNase genes led to the discovery of S-Like RNases in plants, which are not involved in self-incompatibility and are thus found in both self-compatible and self-incompatible plants [16]. Early studies of these enzymes suggest their possible involvement in the remobilization and recycling of phosphate and defense [27].

Arabidopsis thaliana is a member of the Brassicaceae (mustard) family, and is generally considered a weed as it has no economic or agronomic value. However, *Arabidopsis* is an important scientific model organism because it has several useful characteristics: its relatively small genome has been sequenced in its entirely, its life cycle is rapid (4-6 weeks), the plants are relatively easy to cultivate, copious amounts of seeds are produced and are easily acquired, *Arabidopsis* can be efficiently genetically transformed using *Agrobacterium tumefaciens,* and several mutant collections are readily available.

In *Arabidopsis thaliana,* five S-Like RNases have been identified and are named *RNS1, RNS2, RNS3, RNS4,* and *RNS5* [41; 4; Gustavo MacIntosh unpublished]. Very little information is known about RNS4 and RNS5, but more studies have been done involving the other three RNases [3]. RNS1 is known to be secreted extracellularly [5] and involved in wounding responses [27]. RNS3 is predicted to be secreted extracellularly as well. Expression patterns of *RNS1* and *RNS2* hint at their involvement in nutrient recycling [41, 4]. I focused my work on further characterizing RNS2.

What is previously known about RNS2? It is highly expressed in all parts of the plant: roots, shoots, flowers, and leaves, and throughout all developmental stages. Analysis of the RNS2 amino acid sequence suggested that a secretion signal peptide is found in the N-terminus, which implied that RNS2 is part of the secretory pathway. It also suggested that the C-terminus sequence is similar to vacuolar-targeting signals in plants [41]. RNS2 is known to be retained intracellularly unlike other S-Like RNases, and preliminary analyses have suggested that it could be localized to the vacuole or the ER [5].

RNS2 may help remobilize phosphate when that nutrient’s accessibility has become limited, or during senescence. During both senescence and phosphate starvation,

RNS2 expression levels are seen to increase in leaves and petals [41]. Stress in plants can cause anthocyanins to accumulate, which create a purplish color throughout the plant. It has been seen in antisense *RNS1* and antisense *RNS2* plants that there are high levels of anthocyanin accumulation when compared to wild-type, which suggests that these enzymes perform important functions that are not compensated for by other proteins [5]. Also, there currently is no evidence that RNS2 is or is not involved in defense against pathogens. It will be necessary to obtain a knock-out of *RNS2* to use in my experiments to make sure that these results are indeed due to *RNS2*.

In the tomato species, *Lycopersicon esculentum*, *LX* and *LE* are two genes that are also T2/S-Like RNases and are similar to *RNS2* in sequence. Studies have shown that they may share similar functions to that of RNS2. LX affects senescence rates in tomato plants, plays a role in leaf abscission, and KO-*lx* mutants accumulate anthocyanins under phosphate limiting conditions [29; 28].

Because T2 RNases are highly conserved throughout all the kingdoms of life, they may have important roles. By figuring out how these enzymes work in *Arabidopsis*, we can then perhaps apply this knowledge to more economically and agronomically important plants. Since there is limited knowledge in regards to RNS2, I wanted to further characterize it by determining:

1) Does RNS2 play a role in *Arabidopsis thaliana* during phosphate starvation?
2) Within *Arabidopsis* cells, where is RNS2 localized?
3) What is the function of the *RNS2* homologue found in *C.elegans*?

To address these questions, I performed experiments in which I:

a) performed RNase activity assays to determine if RNS2 was functional and indeed absent in the knock-out mutant;

b) observed differences between wild-type, AS-*RNS2*, and KO-*rns2* plants' roots when grown on media with and without phosphate, and media in which RNA is the only source of phosphate

c) determined the anthocyanin content of wild-type, AS-*RNS2*, and KO-*rns2* leaves during times of phosphate starvation

d) grew wild-type and KO-*rns2* plants on media with phosphate, without phosphate, and with RNA as an only source of phosphate to determine if *RNS2* was necessary for phosphate recycling

e) compared wild-type, AS-*RNS2*, and KO-*rns2* plants' leaves during natural and artificial senescence

f) infected wild-type, AS-*RNS2*, and KO-*rns2* plants with TuMV (turnip mosaic virus) as a first attempt to determine whether or not RNS2 is involved in pathogen defense

g) attached CFP to RNS2 protein to visually track the location of RNS2 within *Arabidopsis* cells

h) used RNAi techniques and GFP to observe if CeRNS has any effect on viability, growth, and RNAi rates in *C. elegans*

After performing these experiments I obtained preliminary data that suggest that:

i) RNS2 is indeed functional in wild-type plants and its activity is absent in the KO-*rns2* plants

ii) during times of phosphate starvation (stress), root architectural changes can be observed in AS-*RNS2* and KO-*rns2* plants, which include increased lateral root length and number and decreased primary root length

iii) during phosphate starvation, anthocyanins accumulate, but compared to wild-type, KO-*rns2* plants do not seem different, but AS-*RNS2* plants accumulate twice the amount of anthocyanins

iv) RNS2 is possibly involved in phosphate recycling during times of low accessibility to phosphate and may be involved in flowering time when using plants of a crossed (ColxWS) background

v) RNS2 does not appear to play a role in either natural or artificial senescence, because compared to wild-type, neither AS-*RNS2 n*or KO-*rns2* plants show any differences

vi) RNS2 does not appear to be involved in defense against pathogens (TuMV)

vii) CFP-RNS2 is localized to specialized structures of the ER, known as ER-bodies

viii) *CeRNS* has no apparent effect on rates of RNA interference in *C.elegans*, and is not critical for survival or reproduction under normal growth conditions

CHAPTER II: RNS2 CHARACTERIZATION

INTRODUCTION

When a gene is first discovered, it is important to begin to ascertain its regulation because these findings may lead to determining its main function and other subsequent roles. Having some knowledge about its expression patterns could make the first exploratory experiments yield more definitive results. Because some analysis has previously been done involving *RNS2*, I could continue in the pursuit of discovering more of its characteristics.

Phosphate is found in nucleic acids (sugar phosphate backbone) and the main energy storage molecule (ATP). Thus, it is essential for life and proper plant development and the lack of phosphate will limit plant growth. However, phosphate concentrations found in soils can be very limiting, and plants must adapt to these conditions by recycling internal sources of phosphate, and biochemically extracting more phosphate from the soil and/or expanding the reach of the roots to gain new access to phosphate [36].

During times in which phosphate is in short supply, *Arabidopsis* roots undergo architectural changes, such as increasing the number of lateral roots, the lack of continued growth of the primary root, and increased lateral root length. This is because phosphate availability decreases with soil depth and the plant is more likely to acquire phosphate if it roots are sent throughout the soil near the surface [47]. In addition to morphological changes, plants can adapt to phosphate limiting conditions chemically, by secreting phosphatases and/or nucleases through the roots to help scavenge for organic sources of phosphate. Also, the production of high-affinity phosphate transporters may increase to help bring the essential nutrient into the plant [3; 42].

It is important for flowering plants such as *Arabidopsis* to sense when conditions are favorable to transition from vegetative state to reproductive state, otherwise their reproductive fitness will be reduced. The flowering time of plants can be altered by such things as: vernalization (exposure to cold temperatures before warmer temperatures), day length, light quality, heat, drought, and nutrient deficiency. *Arabidopsis*' vegetative state is characterized by the development of rosette leaves and the absence of a stem. The transition to flowering is marked by the suppression of further rosette growth and the initialization of the growth of the stem and flowers. *Arabidopsis* can be made to flower more quickly when the seeds experience vernalization, and the plants are grown under long day lengths [39].

Senescence is a particular process of programmed cell death, in which the unwanted/unneeded cell(s) undergo reorganization, recycling and reclamation of important nutrients and molecules, and eventually die. This process is natural, although it can be influenced by environmental and/or hormonal changes. Senescence in *Arabidopsis* is most obviously observed by the yellowing of leaves and stems after its reproductive stage has been established. This is because the nutrients within the leaves are being sent to the flowers for reproductive uses. Because the leaves are no longer needed, the chlorophyll is degraded, hence the yellowing color [7].

RNase activity can be quantified and characterized by performing an RNase activity gel assay. This procedure allows RNases to degrade RNA within an acrylamide matrix, and when stained for RNA, any negative bands seen denote an active RNase. This procedure has been routinely performed to identify RNases in *Arabidopsis* [50], but the band associated with RNS2 is currently unknown.

To analyze RNS2 further, I wanted to know:

a) if RNS2 is inactive in KO-*rns2* plants and if I could identify which band corresponds to RNS2 in an RNase activity gel

b) how root architecture is affected in KO-*rns2* plants during times of phosphate starvation

c) if KO-*rns2* plants accumulate more anthocyanins during times of stress than when compared to wild-type plants

d) if RNS2 is involved in phosphate recycling during times of phosphate starvation by comparing phenotypic differences between the wild-type and KO-*rns2*

e) if RNS2 affects the rate of senescence (natural or artificial)

f) if RNS2 is involved in defense against virus infection

RESULTS

Except when stated otherwise, all plants have the Columbia genetic background, and Columbia is the wild-type ecotype. Ecotypes all belong to one particular species, but have adapted to specific habitats and their genomes are slightly different between each other. This variability between genomes needs to be considered when conducting experiments that are utilized to observe morphological differences.

VERIFICATION OF rns2 *KNOCK-OUT MUTANT*

The *RNS2* gene in *Arabidopsis thaliana* can be specifically referenced using its unique locus identifier according to The Arabidopsis Information Resource (TAIR7 release): At2g39780 [2]. A particular knock-out mutant line was created by the Salk Institute using T-DNA. One mutant line that has a T-DNA insertion in the *RNS2* gene is

"*rns2-2"* (Salk #069588), and this line was verified by Danielle Ebany via PCR and sequencing. This particular mutant is referred to as "KO-*rns2*".

First, I wanted to verify that the KO-*rns2* line was indeed a knock-out mutant and was not expressing any functional RNS2 RNase. To accomplish this, I extracted protein from leaf samples taken from 4-week-old wild-type, AS-*RNS2* (antisense *RNS2*, described in [5]) and KO-*rns2* plants. I then performed an RNase activity gel assay. This works by mixing RNA with acrylamide during the preparation of a PAGE gel, running extracted protein through the gel, then renaturing the RNases to activate them. Any active RNases will degrade the RNA in their close proximity. The gel is then stained blue to detect the presence of RNA, and thus a "white band" is a negative stain for RNase activity. As can be seen in FIGURE1, the KO-*rns2* mutant lane is missing an obvious band that the wild-type lane contains. RNS2 is now attributed to this band. This corresponds to a band that is consistently observed in all tissues during all developmental stages, which is consistent with gene expression patterns of RNS2. The AS-*RNS2* sample shows that it still retains an RNS2 band, but the bands were never quantified (as this assay is mainly qualitative).

To show that an equal amount of protein was loaded into the gel, a Coomassie Blue stained SDS-PAGE was performed for verification, see FIGURE2. The 3 lanes show equal loading of samples Columbia, AS-*RNS2*, and KO-*rns2*, each containing 10ug of protein. Three RNA activity gels were created using a single sample for each Columbia, AS-*RNS2*, and KO-*rns2*. These gels have shown that our KO-*rns2* is indeed a knock-out.

PHOSPHATE STARVATION

H_o : *RNS2* is not required to recycle phosphate during times of phosphate starvation in *Arabidopsis thaliana.*

H_a : *RNS2* is required to recycle phosphate during times of phosphate starvation in *Arabidopsis thaliana.*

In order to reject the null hypothesis, we should observe that the KO-*rns2* plants are not viable under less-than-optimal phosphate conditions. Or if RNS2 is not critical for survival during phosphate starvation, the knock-out mutants could show phenotype changes, which may include the typical signs of stress, namely more root lateralization, anthocyanin accumulation, and be smaller in size.

ROOT ARCHITECTURE

The first strategy to test this hypothesis was to grow wild-type, AS-*RNS2*, and KO-*rns2* plants on normal growth media containing phosphate and media that was otherwise normal but lacked any source for phosphate. These seedlings were grown on Petri plates, and all were grown initially on +phosphate plates, and then transferred to +phosphate or –phosphate plates. This experiment was done to observe any visible phenotype differences between the plant lines.

On +phosphate media, there was visibly no difference between the plant lines as all the plants looked like the wild-type. When grown on media without phosphate, all the lines generally looked worse. All lines had more purple-colored leaves (anthocyanin accumulation indicative of stress), and had shorter primary roots but more lateral root growth. The AS-*RNS2* and KO-*rns2* plants suffered more acute phenotype changes compared to the wild-type, in that the plants were smaller and the lateral root growth was

more severe. These results were recorded as general observations, but these seedlings were not individually counted or statistically analyzed, and should be considered to be preliminary results.

This experiment was then repeated but the seedlings were transferred to vertically oriented plates so measurements of roots could take place. On +phosphate media, all the lines of plants had an average primary root length of 3.5cm, but on -phosphate media, the AS-*RNS2* and KO-*rns2* roots were 1-2cm in length, and the wild-type primary roots measured 0.5-1cm in length. This experiment would need to be repeated at least three times to gather enough data to determine if these differences are statistically significant.

ANTHOCYANIN CONTENT

2 week old wild-type, AS-*RNS2,* and KO-*rns2* seedlings that were grown on media containing phosphate and seedlings grown on media without phosphate as described in the previous section were collected. Their anthocyanin content was analyzed, see FIGURE3. On +phosphate media, it was shown that the anthocyanin content of all 3 lines was low and the lines were not significantly different from each other. However, on the –phosphate media, wild-type and KO-RNS2 plants had the same amount of anthocyanins, but the AS-*RNS2* plants had two times more anthocyanin accumulation than the wild-type. Because this experiment was performed only once, it would need to be repeated at least three times to provide enough data to determine statistical significance.

GROWING *ARABIDOPSIS THALIANA* ON +RNA/-Pi GROWTH MEDIA

It is known that *Arabidopsis* can be grown on media where nucleic acids can be substituted for a source of phosphate [42]. It is also known that both *RNS2* and *RNS1* genes are induced when phosphate is not readily accessible from the media/soil [5].

Because I do not know whether *RNS1* can compensate for *RNS2* function, I decided it was necessary to obtain double-homozygous knock-out mutants for both genes (dhms). The *rns1* mutants were obtained from the University of Wisconsin and isolated and verified by Nicole LeBrasseur and Gustavo MacIntosh. An important difference is that this mutant line was created in the WS ecotype. I crossed *rns1*(WS) (male) with *rns2*(Col) (female) plants to create the dhms, and also crossed wild-type Columbia plants with wild-type WS plants to be used as a future control plant line. From these crosses, I also obtained new *rns1* and *rns2* homozygous mutants (with a ColxWS genetic background).

However, because our KO-*rns1* plants have an apetala phenotype that is unrelated to RNS1, obtaining enough crossed progeny was difficult. Eventually via PCR genotype verification, dhms were initially identified but few seeds were collected. Another problem was cross-pollination contamination within the supposed dhm lines because further PCR tests showed that wild-type DNA was present in these “dhm” plants. Thus, any results involving the dhm plants were excluded from these results due to their genetic contamination. It was with the following 5 different lines in which I continued my phosphate starvation experiments: Columbia, WS, ColxWS, *rns1*(ColxWS), and *rns2*(ColxWS). Each plant line used from the ecotype cross is an independent verified line.

Instead of using Petri plates I decided to use Magenta boxes to allow the plants more room to grow. The RNA used as the source of phosphate was made using purified Torula Yeast RNA. I then grew the 5 lines of plants in Magenta boxes containing normal media (+phosphate) and media with RNA as its only source of phosphate (+RNA/-Pi). No plants were grown on –phosphate media seeing as those plants do not grow well, and transferring seedlings from +phosphate plates would be necessary to allow the seeds to

initially germinate, and this would cause great contamination problems within the Magenta boxes.

After 2 weeks, all the plants grown on +phosphate media looked normal (including WS looking to be slightly larger in size), but the plants grown on +RNA/-Pi media were all comparatively diminutive in size and slightly purplish in color (anthocyanin accumulation). After 4 weeks, it was noticed that many of the plants grown on +phosphate media had bolted or had begun to enter the reproductive stage (ie, bolt), but many plants on the +RNA/-Pi media had not. From that point on, plants were counted per Magenta box per treatment and per plant line, and it was recorded how many had begun to bolt. Boxes were counted every three days, and plants were considered to have bolted if its stem was at least 1.25cm tall.

It can be seen from FIGURE4 that after 4 weeks on +phosphate media almost all the WS, ColxWS, *rns1*(ColxWS) had flowered, but less than 20% of the Columbia and none of the *rns2*(ColxWS) plants had bolted. It took approximately 5 weeks for more than 90% of the Columbia to have bolted, and even by week 6 only slightly more than half of the *rns2*(ColxWS) plants had begun to bolt.

The differences for bolting were even more dramatic when viewing the data on the +RNA/-Pi graph, FIGURE4. All lines of plants were delayed in their flowering times compared to their +phosphate counterparts. The percentage of flowering plants over weeks 4-6 climbed steadily for all the lines of plants except noticeably, the *rns2*(ColxWS) plants, which did not flower. Actually, *rns2*(ColxWS) plants were observed to show 50% bolting by week 9, but the bolts were very short, <= 2.5cm (data not shown). It should also be noted that the *rns2*(ColxWS) plants have much larger rosettes with more and larger leaves, consistent with the phenotype observed for other flowering-time (FT) mutants [43].

The same results were seen when these 5 lines of plants are grown on soil; the *rns2*(ColxWS)'s flowering time is delayed compared to all the other types of plants, including its control ColxWS, FIGURE5. These results were different for the KO-*rns2* mutant that only had the Columbia genetic background. FIGURE6 shows (left to right) KO-*rns2,* AS-*RNS2,* and Columbia plants (4 weeks old) grown on soil and there is no significant difference in flowering time among these lines.

PHOSPHATE CONTENT

Phosphate content (inorganic phosphate) analysis was performed by Collin Bakkie and Ludmila Rizshsky using the material provided by the experiment described in the previous section. It is included in this thesis because the results add to the discussion on the role of RNS2. This data was to determine if the lack of *RNS2* or *RNS1* played a role in inorganic phosphate uptake and/or internal usage.

All plants of the same line grown on the same media were combined into one sample and ground up, and then the inorganic phosphate was extracted. According to FIGURE7, it was observed that all the lines of plants grown on the +phosphate soil contained approximately the same amount of Pi. However, when grown on +RNA/-Pi media, there was greater variability among the lines. Comparing between the single mutants and the appropriate control (ColxWS), it was observed that *rns1*(ColxWS) has significantly less Pi and *rns2*(ColxWS) has significantly more Pi than ColxWS.

SENESCENCE

To determine if RNS2 has a function during natural senescence, I grew wild-type Columbia, AS-*RNS2* and KO-*rns2* plants on soil for 8 weeks to observe any phenotypic changes during natural senescence. After several independent trials, no significant differences were seen between the plant lines in regards to natural senescence rates.

However, when *rns2*(ColxWS) plants were grown on soil, they exhibited delayed natural senescence compared to the control, ColxWS, probably due to the delay in flowering.

To ascertain if KO-*rns2* plants experienced a different phenotype while being subjected to artificial senescence, I obtained 10 random leaves from 4 week old plants from each plant type and created an artificial senescing environment, in which the detached leaves were kept undisturbed at room temperature in the dark on moist filter paper. I photographed the senescing leaves 0, 3, 7, and 10 days after being cut from the plant to record the changes of the leaves, see FIGURE8. After the leaves were photographed, they were ground up and analyzed for their chlorophyll content, a more quantitative way to assess the rate of senescence. After 4 independent trials, however, it was observed that there were no differences in the rate of senescence between the 3 lines of plants.

DEFENSE AGAINST VIRUS INFECTION

To determine if KO-*rns2* mutants are more susceptible to virus infection than wild-type plants, I grew Columbia, AS-*RNS2* and KO-*rns2* plants on soil. When the plants had bolts 4-5” tall, they were infected with TuMV, which is known to infect *Arabidopsis* plants [45]. The TuMV also had constitutive GFP expression so infection could be observed. UV light is used to view the plants so that any uninfected tissue would appear red under UV light, and any infected tissue will look fluorescent green. After 6 days post-infection, the plants were viewed everyday under UV light for any infected apical tissue, verifying the virus infection had spread systemically.

Preliminary experiments performed by Melissa Hillwig suggested that virus infection spread faster to the apical tissue in KO-*rns2* plants than when compared to wild-type. However, in my experiment no differences were seen among any of the three lines

of plants for rate of virus infection, FIGURE9. No obvious differences in the intensity of the infection were observed either. (17 days post infection, any plant that became infected was almost-entirely to entirely infected (data not shown)).

DISCUSSION

First, it was important to establish that the KO-*rns2* mutant completely lacks RNS2 RNase activity because any conclusions that were drawn based upon this fact would be more substantial. It was also important to establish which band was attributed to RNS2 within the RNase activity gel assay profile because that standard had not been previously established. Because RNS2 is always expressed, the lack of a band obviously indicated a knock-out mutant. The RNase activity in the AS-*RNS2* line should have been absent or certainly less than that observed in the wild-type, and although it may look to have the same activity, the amount of activity was not quantified. In conclusion, it was determined that the KO-*rns2* line is indeed has *RNS2* knocked-out, and the band associated with *RNS2* was determined within RNA activity gels. It is also clear that the AS-*RNS2* still shows a band for *RNS2*, indicating that it is not a null RNS2 mutant.

The increase of lateral root growth and the decrease in length of the primary root are responses to gain access to phosphate to alleviate the starvation condition. If *RNS2* was involved in internal phosphate recycling and an outside source of phosphate was scarce, we would expect the KO-*rns2* plants to have more severe changes in root architecture compared to the wild-type. But this was not observed, and in fact the AS-*RNS2* and KO-*rns2* plants had slightly longer primary roots on average when compared to the wild-type. However, because these plants were not analyzed statistically and only one trial was performed, these are only preliminary results. Additional trials with

statistical analysis would be necessary to determine if there are any significant differences in root architecture between the plant lines.

During times of stress, anthocyanins can accumulate, and it is assumed that the harsher the stress, the more anthocyanins will accumulate. To determine relative amounts of stress, the anthocyanins can be extracted from the leaves and quantified. It was observed that when subjected to phosphate starvation, the wild-type, AS-*RNS2* and KO-*rns2* lines all showed large increases in anthocyanin accumulation, but the AS-*RNS2* line exhibited twice the accumulation compared the other two lines. This is surprising because it would be expected that the knock-out mutant line would accumulated the most anthocyanins. This type of result has been seen once before, in AS-*RNS1* plants when compared to wild-type and KO-*rns1*,[26] thus this effect may be an artifact of the antisense implementation.

Having a double-homozygous mutant line of plants (*rns1rns2*(ColxWS)) would have helped rule out the possibility that *RNS1* may be compensating for *RNS2*. However, nothing substantial can be drawn about these plants because after trying to verify their genotype, it was observed that wild type DNA was present in the putative double homozygous mutants. Thus, any data pertaining to the dhm plants was left out of this thesis because any subsequent conclusions based upon this data would be false.

The startling phenotype difference seen in regards to flowering time in the *rns2*(ColxWS) line which is exacerbated during times of phosphate starvation, clearly supports the idea that *RNS2* could be involved in phosphate starvation responses. Although it can acquire phosphate from the media, as can be seen via the Pi content levels, internally, it may only have access to the inorganic form because it is unable to recycle its organic phosphate. Additional trials of this experiment and the use of more genetic controls would be very helpful to verify the RNase's role.

To determine more definitively whether or not RNS2 is responsible for the late flowering phenotype (LF), a better designed experiment would be necessary. One of the problems with the experiment that was carried out was the lack of controls that gave useful information and there were not enough independent *rns2*(ColxWS) lines to distinguish between epigenetic influences and RNS2's role in the LF phenotype. I will outline a better designed experiment here with interpretation of possible results, and will assume there are no technical problems/difficulties with the procedures, recipes, etc.

H_o : RNS2 is not responsible for the late flowering phenotype

H_{a1} : RNS2 is responsible for the late flowering phenotype

To reject the null hypothesis, enough independently-isolated *rns2*(ColxWS) lines would need to be grown and shown to have LF. And then a complementation experiment would need to show that RNS2 is indeed responsible for LF.

First, at least 30 plants of each 6 independently-isolated *rns2*(ColxWS) lines would need to be grown in Magenta boxes. To ensure independence of the lines, each line will be the result of an isolated cross. In addition, Columbia, WS, and ColxWS control plants would be grown the same way. This is the first step to determine RNS2's role in late flowering. One possible result is all the *rns2*(ColxWS) lines are late to flower; this is a strong indication that *RNS2* is responsible for the late flowering phenotype. However, if not all the lines show late flowering, then possibly there are epigenetic interactions between Columbia and WS that are causing LF, or these interactions are affecting RNS2.

To really determine what is causing LF, a complementation experiment needs to be performed. The complementation experiment should use any *rns2*(ColxWS) plant lines that exhibited late flowering (at least 5 independent lines). Each of these lines would be subjected to three treatments: 1) no manipulation, 2) transformed (complemented)

with RNS2 (using a plasmid containing RNS2), 3) transformed with an empty vector. Then these plants would be grown in Magenta boxes as normal. The results I would expect would be: 1) that any untransformed plants would still exhibit the late flowering phenotype; 2) plants that were complemented with RNS2 would return to a normal flowering phenotype; and 3) plants that were transformed with an empty vector would still exhibit LF. These expected results would indicate that RNS2 is indeed responsible for LF. However, if all or some of the plant lines that were complemented show a late flowering phenotype, then I would know that epigenetic factors are causing LF, and RNS2 is not responsible for LF. Of course, northern blot or RT-PCR analyses should be performed to ensure that *RNS2* is expressed in the complemented lines. Also, if plants that were transformed with the empty vector revert back to having a normal phenotype, this would also indicate RNS2 is not responsible and epigenetics are at work.

From my results, it cannot be concluded that *RNS2* has any involvement in senescence, although previous data has shown that expression levels rise during senescence. But *rns2*(ColxWS) plants did exhibit delayed natural senescence when grown on soil in comparison to the control. This is probably due to the fact that it flowered later than the control. It may be necessary to perform the senescence experiments again using the *rns2*(ColxWS) mutant because that particular line showed a very distinctive phenotype difference during phosphate starvation experiments, and therefore may show a different phenotype for senescence rates. This may be difficult to analyze however, because if this line of plants flowers late, it will senesce later compared to the wild-type plants. This problem may be circumvented if senescence rates are counted once a plant has reached a particular age within the reproductive stage, or using induced artificial senescence (detached leaves experiment).

It does not seem that *RNS2* has any involvement in defense against virus infection, despite the preliminary observations from Melissa Hillwig. However, this experiment was only performed once and perhaps more trials are necessary, or the use of *rns2*(ColxWS) would prove to have more interesting results. Also, only one type of pathogen was used, but others could be tested.

Because there is not enough evidence that *RNS2* is required to recycle phosphate, I cannot reject the null hypothesis at this time.

CONCLUSIONS

KO-*rns2* mutant plants show signs of phosphate starvation such as accumulation of anthocyanins and increased lateral root growth and shortened primary roots, but when compared to the wild-type phenotype under the same conditions, there is no apparent difference. KO-*rns2*(ColxWS) exhibited delayed flowering times when grown on normal media and even more extremely delayed flowering times when grown on media where RNA was the only source of phosphate. This line of plants also shows increased inorganic phosphate content when grown on +RNA/-Pi media. KO-*rns2* mutants and AS-*RNS2* plants did not show any differences in natural or artificial senescence rates when compared to wild-type plants. Also, KO-*rns2* and AS-*RNS2* did not show any different infection rates when challenged with TuMV when compared to the wild-type.

After further characterizing *RNS2* in *Arabidopsis thaliana*, it is unclear whether or not *RNS2* has any noteworthy effects on plant growth when phosphate conditions are less than optimal. These results do show that *RNS2* is not necessary for plant viability, but may be necessary for proper phosphate recycling and/or normal flowering times. As mentioned before, a more thorough experiment would need to be conducted to yield

conclusive results. Also, *RNS2* does not appear to be involved in natural or artificial senescence, and is not involved in defense against TuMV infection.

FUTURE EXPERIMENTS

As mentioned previously, a more thorough experiment would need to be conducted to test whether or not *RNS2* is involved in flowering time delays. Other experiments that are currently underway or could be conducted in the future to further characterize RNS2 in *Arabidopsis thaliana* include: growing the plants on +phosphate and +RNA/-Pi vertical Petri plates to determine differences in root lengths and architecture, and anthocyanin content; growing the plants on +phosphate and +RNA/-Pi media to analyze inorganic phosphate content; growing the plants on soil for phenotypic differences including natural senescence, artificial senescence, and seed production.

Other experiments would include: to determine if RNS3, RNS4, and/or RNS5 proteins are compensating for RNS2 by creating double mutants and growing on +RNA/-Pi media, and to see if *RNS3*, *RNS4*, and *RNS5* are expressed during phosphate starvation; to determine if phosphate transporters are being affected in *rns2* mutants; to introduce *RNS2* back into the knock-out mutants to see if the wild-type phenotype is reverted (complementation); and to use *rns2*(ColxWS) plants in virus defense experiments.

CHAPTER III: RNS2 LOCALIZATION

INTRODUCTION

Because plants cannot move, they must be able to adapt to their environment, and have developed ways to aid their reactions to stress. It has been observed that the endoplasmic reticulum within plants have become very specialized to perform certain functions to allow the plant to adapt. The endoplasmic reticulum (ER) is found in most types of cells and basically functions as a protein transport and packaging system. In addition to the expected tube and membranous sheets found in normal ER composition, plants also create specialized compartments derived from the ER to assist during specific environmental or developmental stresses, known as ER-bodies [20]. These compartments can store proteins and oils, enzymes for responding to pathogen attacks, and particular proteases which are used to degrade seed storage proteins during germination. Most ER-derived protein bodies bud from the cis-ER but are still enclosed in ER membranes, and can either remain in the cytosol or become part of a vacuole [14]. Certain features of ER-bodies imply that these compartments have specialized jobs: 1) they accumulate large amounts of a single or a few kinds of proteins, 2) the proteins do not function within the compartment, and 3) the bodies appear in specific tissues during specific times [18].

In *Arabidopsis*, ER-bodies are not found throughout all tissues at all times, and thus shows how plants can adapt during times of change. For example, in cotyledons ER-bodies appear after germination but disappear during senescence. ER-bodies are not normally found in rosette-leaf cells but appear after wounding. In addition, appearance of ER-bodies can be under hormonal control, ie) methyl jasmonate (is induced during times of pathogen-attack and wounding) and ethylene (suppresses MeJA) [18].

To better understand an enzyme's function, a critical piece of information would be to know its location within a cell. Knowing in which organelle or system a protein can be found may quickly increase our idea of what roles that protein may play and in which pathways it may participate. From looking at the N-terminal sequence of amino acids encoded by the *RNS2* gene, it was thought that there is a secretion-signal (19aa) that directs RNS2 into the secretory pathway [41]. However, RNS2 is kept intracellular [5], and at the C-terminus is a putative ER-retention signal (R-E-A-L) that could potentially keep the RNase within the ER, although vacuolar localization for RNS2 has been proposed [41]. By verifying that RNS2 is within the secretory pathway and stays within the ER or is associated with vacuoles, we could then better understand how it may interact with RNA and the possible roles it could play during phosphate starvation.

I wanted to know exactly where RNS2 is localized within *Arabidopsis* cells. In order to view the location of RNS2 within cells, I created a construct in which CFP (cyan fluorescent protein) was fused to the RNS2 protein and was then transformed into *Arabidopsis* plants. Using confocal microscopy, the CFP could be seen and it would be assumed that wherever CFP was located RNS2 would be found.

RESULTS

H_o : RNS2 proteins not localized to either the endoplasmic reticulum or the Vacuole in *Arabidopsis thaliana* cells.

H_{a1} : RNS2 proteins are localized to the endoplasmic reticulum in *Arabidopsis thaliana* cells.

H_{a2} : RNS2 proteins are localized to the vacuole in *Arabidopsis thaliana* cells.

To reject the null hypothesis, there needs to be a positive verification that RNS2-CFP is found within either (or both) the endoplasmic reticulum or the vacuole.

CFP-RNS2 LOCALIZATION

To determine where RNS2 was being localized within *Arabidopsis* cells, the following steps were taken: 1) a CFP-RNS2 construct was created, 2) *Agrobacteria* were transformed with the CFP-RNS2 construct, 3) *Arabidopsis* Columbia plants were transformed with the *Agrobacteria*, 4) progeny from the transformed plants were screened for the expression of transgenes (using the selective marker kanamycin), 5) protoplasts were prepared from the verified progeny and viewed under a confocal microscope, 6) root tissue was viewed under a confocal microscope.

The RNS2 protein includes a 19 amino-acid secretion-signal at the N-terminal, and a putative ER-retention signal (R-E-A-L) at the C-terminal end. Secretion-signals are normally cleaved off during transport through the secretory pathway. If CFP was attached to the N-terminal end of RNS2, it would likely be cut off along with the secretion signal and then RNS2 could not be tracked, or CFP would disrupt the proper secretion of RNS2 and it would be found in the cytoplasm and kill the cell. If CFP was attached to the C-terminal end, it could disrupt the putative ER signal. To avoid this problem, CFP was inserted between the 19^{th} and 20^{th} amino acid (after the secretion signal) to create the CFP-RNS2 construct. In addition, a CFP-only plasmid was used as a control to view CFP expression in comparison against the wild-type autofluorescence and as a marker for cytoplasmic localization. In both constructs, a 35S promoter was used to ensure high levels of expression, see FIGURE10.

Using *Agrobacteria* is an easy and effective way to genetically transform *Arabidopsis* plants, and would allow for expression of the constructs. Both plasmids that contained the CFP-RNS2 construct and CFP-only also contained an antibiotic resistance gene (kanamycin), so plants that had taken up the construct could be screened. Kanamycin resistant plants were transferred to soil. When these plants produced flowers,

their petals were viewed under a fluorescence microscope for CFP expression. Untransformed wild-type plants were used as visual controls.

Several CFP-only plants were verified to have CFP expression, and 5 CFP-RNS2 plants were verified to have CFP-expression in the petals as well. CFP-only petals appeared very blue compared to the wild-type's autofluorescence, and the CFP-RNS2 petals were less blue than CFP-only, but significantly more blue than the wild-type. However, it should be noted that CFP-RNS2 plant #5 seemed to have particularly blue petals, equivalent to CFP-only petals. CFP-RNS2 plants #2 and #3 seemed to have the next most CFP expression, and plants #1 and #4 had less CFP expression but still more than just the autofluorescence seen in wild-type petals.

Viewing transgenic protoplasts would be a good way to verify that indeed CFP was being expressed within the plant cells, and to perhaps view where RNS2 was being localized. Protoplasts were prepared from wild-type and both types of transformed plants and viewed with Robert Doyle's assistance using a hyperspectral microscope, FIGURE10. The results show that RNS2 is in a particulate structure, "dots", which is consistent with endomembrane localization. However, the resolution of this microscope was not enough for conclusive localization.

Seeds from Columbia, CFP-only, and CFP-RNS2 #2, #3 and #5 were sent to the imaging facility of the Delaware Biotech Institute to obtain higher quality localization images. The seeds were grown on normal growth media, and the root tissue of 2 weeks old seedlings was viewed under a confocal microscope. These pictures proved to be more informative, FIGURE11. It can be seen that the wild-type shows only low autofluorescence, and the CFP-only root tissue is expressing CFP throughout the cytoplasm. In contrast, in both CFP-RNS2 #2 and #3 root samples exhibit CFP expression in very distinct rice grain-like structures that resemble ER-bodies. For CFP-

RNS2 #5 (data not shown), CFP was expressed throughout the cytoplasm and was not found in ER-bodies, similar to the CFP-only localization images.

CFP-RNS2 RNASE ACTIVITY

To determine if the CFP was affecting RNase activity of RNS2, I performed an RNase activity gel assay, FIGURE12, using protein extracts from each of the 5 CFP-RNS2 plant lines, a CFP-only plant, and wild-type Columbia plant. If the RNase was still active, it would degrade the RNA nearby and after staining for free RNA, a “white” band could be seen. In FIGURE12, it is shown that the 7 protein extracts were run through the gel, and indeed the RNase still had activity while attached to CFP. Also, there was a large increase in RNS2 activity in CFP-RNS2 #2, #3, and #4 when compared to wild-type. While level of activity of #1-4 correlates with CFP intensity, #5, the most fluorescent sample, does not show activity corresponding to CFP-RNS2, nor does it show an increase in RNS2 activity. An RNase activity gel was performed twice, but used the same samples taken from the transgenic plants. A Coomassie gel was performed and showed that these differences were not due to unequal loading of protein (FIGURE13).

DISCUSSION

The confocal microscopy pictures show where CFP is detected. In the wild-type picture, only a minimal amount of autofluorescence is seen. The CFP-only picture verifies that *CFP* is being expressed and the protein is localized within the cytoplasm (the vacuole takes up most of the volume of the cell, and thus pushes the cytoplasm to the sides) and nucleus. In CFP-RNS2 lines #2 and #3, it is very obvious that CFP is being localized to the distinctive-looking ER-bodies.

It was assumed that wherever CFP was visualized, RNS2 protein would be in the same place. There is no other structure within cells that looks the same way ER-bodies

do, and it is very obvious that CFP is seen within these compartments. Thus RNS2 is being localized to ER-bodies. Because ER-bodies are known to aid the plant during times of stress, this locality adds to the idea that this RNase is involved in some emergency response. However, colocalization of CFP-RNS2 and an ER-body marker should be performed to confirm this.

The RNase activity gel verifies that while attached to CFP, RNS2 retains its RNase activity. In addition, endogenous RNS2 shows a substantial increase in activity in the CFP-RNS2 lines as well. This may be because in some instances, CFP is being cleaved off RNS2. But because CFP-RNS2 bands are seen, it can be assumed that in the confocal pictures at least some of the CFP is still tracking the location of RNS2. On the RNase activity gel, there are three bands of slightly different sizes seen where CFP-RNS2 is active. This slight difference is sizes may be attributed to glycosylation and RNS2 is known to have two glycosylation sites [41; 3]. Because CFP-RNS2 #5 line does not show any CFP-RNS2 bands in the activity gel and it was observed that CFP was expressed only within the cytoplasm, I hypothesize that in this plant CFP is being cleaved off RNS2 and sent to the cytoplasm. If an active RNS2 protein was localized to the cytoplasm, the cell should die [MacIntosh, unpublished].

The use of a 35S promoter in both constructs may affect the way the cells utilize RNS2. ER-bodies are not always present in all tissues and stages of life in *Arabidopsis*, and it is not clear why ER-bodies are present during the localization RNS2 experiments. While ER-bodies are normally present in *Arabidopsis* seedlings, it is also possible that CFP expression and/or the elevated expression of CFP-RNS2 produces internal stress causing ER-bodies to form, although RNS2 is always expressed at some level in all tissues.

RNS2's C-terminal end sequence suggested that RNS2 could be localized to the ER or vacuole. It could be possible that if RNS2 is being localized to the vacuole, CFP is being cleaved beforehand and RNS2 is not tracked. The large increase of RNS2 activity observed in CFP-RNS2 plants suggest that this could be the case. In addition, a proteomics analysis of *Arabidopsis* vacuoles identified RNS2 as a vacuolar protein [8]. This is consistent with the hypothesis that ER-bodies are part of the vacuolar targeting pathway [20]. Other experiments would have to be conducted, such as vacuolar colocalization with RNS2 and a known vacuolar protein, to determine if any RNS2 protein is being sent to the vacuole.

The null hypothesis can be rejected in favor of the alpha-one hypothesis, because there is convincing evidence that RNS2 is being localized to the ER (ER-bodies).

CONCLUSIONS

In conclusion, RNS2 protein is being directed to ER-bodies within *Arabidopsis thaliana* cells.

FUTURE EXPERIMENTS

To further confirm that RNS2 is being localized to ER-bodies within *Arabidopsis* cells, RNS2 could be colocalized with an ER-body marker. Another experiment would be to isolate ER-bodies from cells, perform a protein extraction, and then use antibodies specific for RNS2 (Western blot) to detect the presence of the RNase. Also, to test the function of the two signals found at either terminal of the protein, each signal could be removed and/or altered to determine that it is indeed the amino acid sequences that direct RNS2 localization.

CHAPTER IV: CeRNS

INTRODUCTION

C. elegans makes a great model organism because it is relatively simple to care for, its generation time is approximately 3 days, its entire genome has been sequenced, each cell's lineage has been determined, and RNA-interference (RNAi) makes it relatively easy to discern unknown functions of genes.

Because T2 RNases are conserved throughout the kingdoms of life, they are thought to play critical roles to maintain proper life functions. Analysis of amino acid similarity between genes of several species indicates that *RNS2* of *Arabidopsis* is most similar to *C. elegans'* only T2 RNase, corresponding to locus WBGene00019624 from WormBase (release WB176, June19, 2007) and sequence K10C9.3; referred to as *CeRNS* from now on. See FIGURE14 for *CeRNS'* cDNA sequence, and FIGURE15 for an amino acid phylogenetic tree. The transcripts corresponding to this region of the genome are annotated differently in a different database (ACEVIEW), however, the accuracy of the annotation present in WormBase was confirmed by RT-PCR. Little is known about this gene, but it is expressed during early stages of embryogenesis [6]. Even though *CeRNS* is similar to *RNS2*, it is unlikely that *CeRNS* plays a role in a phosphate-starvation response because the nematodes obtain the necessary phosphate from the bacteria they consume, unlike plants which need to extract the phosphate from their environment and thus are subjected to large variations in phosphate availability. While many putative functions could be assigned to CeRNS, a particularly interesting one is the idea that *CeRNS* might participates in RNA interference (RNAi), either during establishment or inheritance of RNAi. Since CeRNS is predicted to be expressed extracellularly, and it is possible that

RNA uptake or accumulation of RNA molecules that play a role in systemic spreading of RNA silencing are regulated by CeRNS.

RNAi techniques can “knock-down” functional genes to create temporary loss-of-function mutants. This can be done because cells have a pathway to destroy any double-stranded RNA (dsRNA), and by making cells uptake dsRNA that is complimentary to a particular gene, its expression can be obstructed. Eukaryotic cells have made use of this pathway to hopefully destroy invading viruses. RNAi works through the culmination of several steps: the full dsRNA is cleaved into smaller fragments (22-ribonucleotides long) known as siRNA through the action of Dicer. RISC (RNA-Induced Silencing Complex) unwinds the 22mer to make ssRNA. RISC will continue on with one of the strands of RNA which becomes the guide RNA. The RISC/RNA complex can then bind its target transcript, which results in the degradation of the host mRNA and proteins are never translated, thus gene expression is inhibited [13; 1].

The reason why RNAi is so easily established in *C.elegans* is because its diet consists of eating bacteria. These bacteria can be transformed with a double anti-parallel T7-promoter plasmid in which any desired sequence that is placed between the T7 promoters will be transcribed into dsRNA. When the nematodes eat these bacteria, they will take up the dsRNA and RNA interference will occur. If the dsRNA is similar to a gene found within *C.elegans*, then effective knock-out mutants can be made (known as “knock-down” mutants). Also, RNAi can be passed on to the next generation, allowing for the study of genes that may only be expressed during embryogenesis.

Double-stranded RNA is a signal for a cell to initiate RNAi processes, and because dsRNA can travel between *C. elegans’* cells, this can trigger an RNAi response throughout the entire organism. An RNase could affect the RNAi process, in particular during uptake of RNA or during the transmission of the interference from parent to

embryo. Perhaps *CeRNS* is part of the RNAi network and the rate of RNAi would be altered if *CeRNS* was knocked-down.

To test this idea, I used nematodes that constitutively expressed GFP. I then kept their *CeRNS* expression knocked-down by constantly feeding them CeRNS-RNAi bacteria (I will refer to these nematodes as "knocked-out"). To control for normal RNAi rates, I used *unc-22*-RNAi bacteria in separate nematodes that became knocked-out for *unc-22. Unc-22* encodes twitchin, a giant intracellular protein required in muscle maintenance of normal morphology. Mutant *unc-22* nematodes appear to be constantly twitching (uncoordinated) and have abnormal muscle structure, and so their knock-down phenotype would be apparent [13]. Because this gene is not involved in the RNAi process, I can compare RNAi rates between the two treatments.

To determine any changes in RNAi rates, I induced GFP RNA interference in GFP-expressing nematodes by temporarily feeding the nematodes with bacteria that would knock-down their GFP expression. After 24 hours, I stopped feeding them the GFP-RNAi bacteria, which would allow GFP expression to return. By counting how many nematodes are green before, during, and after being fed GFP-RNAi bacteria, I could determine if knocking-down *CeRNS* has an effect on RNA interference rates in comparison to the *unc-22* control.

RESULTS

H_o : *CeRNS* does not contribute to *C.elegans'* RNAi machinery and will not alter the rate of RNAi if *CeRNS* is knocked-out.

H_a : *CeRNS* is part of *C.elegans'* RNAi machinery and will alter the rate of RNAi if *CeRNS* is knocked-out.

If the null hypothesis is not true, then when compared to KO-*unc-22*, KO-*cerns* nematodes will show an altered rate of GFP-expression recovery, and/or the progeny from the parent nematodes may have altered GFP expression.

GFP RNAi ESTABLISHMENT AND GFP EXPRESSION RECOVERY

To test this hypothesis, I used GFP-expressing nematodes, and subjected them to 4 different RNAi-bacterial treatments: 1) *unc-22*-only to control for induced RNAi effects; 2) *CeRNS*-only RNAi to induced RNAi for CeRNS; 3) *unc-22* RNAi and GFP RNAi bacteria for a control of RNAi rates; 4) CeRNS and GFP bacteria to compare RNAi rate differences between worms with or without CeRNS expression. Each treatment of nematodes was counted for GFP expression after 4, 8, 12, and 24 hours. Then nematodes were transferred to plates without any GFP-RNAi bacteria, and were counted after 12, 24, 48, and 72 hours of recovery. See FIGURE16 for a simplified schematic of this experiment, FIGURE17 to view GFP RNAi, and FIGURE18 to view CeRNS RNAi.

Despite many independent trials, no consistent or significant results could be obtained except that nematodes which were not given GFP-RNAi bacteria were never observed to be white (GFP expression was never lost). FIGURE19 shows representative data of the GFP-expression counted from the nematodes. The rates of green-to-white nematodes would differ between each trial and white-to-green nematode recovery rates would often be confusing. The only conclusion that can be drawn from this experiment is that CeRNS is not required for survival and does not seem to affect reproduction or the progeny, based upon the observation that eggs were still seen and nematodes of all life-cycle stages were seen during this experimental treatment.

RT-PCR VERIFICATION OF RNAi

Verification that RNAi was indeed silencing *CeRNS* was necessary because unlike unc22 and GFP silencing, there was no obvious phenotypic change. Two populations of GFP-expressing nematodes were fed regular bacteria and CeRNS-RNAi bacteria. After a large enough population was attained, RNA from both populations was then extracted, then a reverse-transcriptase procedure created cDNA from the RNA. PCR was used to detect the presence of CeRNS cDNA (FIGURE14) in these two samples and in addition, two control samples, cDNA obtained from non-GFP-expressing nematodes at the adult and embryonic stages. CeRNS cDNA was detected in the two controls samples and in the GFP-expressing nematodes that were fed normal bacteria. No CeRNS cDNA was detected from GFP-expressing nematodes that were fed CeRNS-RNAi bacteria.

DISCUSSION

Despite multiple trials of this experiment, nothing conclusive can be drawn from these observations, except that knocking-down *CeRNS* is not lethal, and does not seem to affect reproduction. Because it was verified that CeRNS silencing was occurring, this was not considered to be the cause of the inconsistent results. The inconsistency of the data may be due to the fact that the amount of GFP was not directly quantified, but subjectively observed. Another problem with the experiment was that the nematodes had to be paralyzed in order to be counted properly, and this required a large number of nematodes for each treatment. Any nematodes that were counted were killed, and thus their progeny were never observed. This led to the problem of not always having enough nematodes at the end of the experiment to be statistically useful. Also, only sometimes a fresh population was used (subsequent from bleaching), and this may have affected the result in unknown ways.

The null hypothesis cannot be rejected because there is no evidence that contradicts the idea that *CeRNS* plays no role in RNAi in *C.elegans.*

CONCLUSIONS

Because there were no consistent significant differences between GFP-expression recovery rates, is unclear as to whether or not *CeRNS* has any effect on RNAi rates in *C.elegans*. But it can be concluded that *CeRNS* is not essential for survival or reproduction.

FUTURE EXPERIMENTS

Future experiments that could be done to analyze the function of *CeRNS* would be to obtain genetic KO mutants of this gene, rather than relying on RNAi for creating knock-downs. Then perhaps a phenotypic change could be observed. Because *CeRNS* is similar to *RNS2* in *Arabidopsis*, experiments could test for involvement in pathogen defense, or coping with phosphate limitation.

OVERALL CONCLUSIONS

RNS2 is an S-Like RNase that needs further characterization and analysis, but it has been shown to have some role in phosphate recycling especially during phosphate starvation conditions and may affect flowering times. Repeats of these experiments and more highly controlled data is necessary to confirm these claims. However, it does not appear that *RNS2* has a role in defense against virus infection, and it is questionable that it has an effect on root architecture or senescence. RNS2 was localized to ER-bodies within *Arabidopsis* root cells using CFP as a visual marker. Because ER-bodies are specialized to help the plant deal with stresses, it further validates that RNS2's role is involved in helping the plant recycle phosphate during times of phosphate starvation, although more experimentation is required to reject the null hypothesis. It cannot be concluded that *CeRNS* has any effect on RNAi rates in *C.elegans*, but it is not essential for nematode survival or reproduction.

MATERIALS & METHODS FOR RNS2 CHARACTERIZATION

CARING FOR PLANTS

Plants that were grown on soil or in Magenta boxes were cared for as described in [41]; they were grown in a growth chamber that maintained the following conditions: long-day cycle (16 hours light, 8 hours dark), maintained a temperature of 21^{oC} , and the relative humidity was kept at 69%.

MEDIA PREPARATION

The +phosphate and –phosphate media was prepared as described in [4]. Normal AGM was prepared as described in [41].

RNASE ACTIVITY GEL and COOMASSIE GEL ASSAYS

Protein extraction buffer was prepared according to [31] and proteins were quantified using a standard Bradford assay.

High molecular weight RNA used for RNase activity gels was purified from torula yeast RNA (Sigma R-6625) using phenol:chloroform:IAA and chloroform extractions. Then the RNA was precipitated out using 1:10 (volume) of 3M NaOAc at pH 5.0 and 2 volumes of ethanol. After centrifugation, the RNA was briefly washed with 70% ethanol, and put into a lyophilizer for 3 days. Finally, the RNA was dissolved in 0.1M Tris pH 8.0 and quantified.

RNase activity gel assays were performed according to [50].

Proteins were visually quantified using a Coomassie-blue-staining procedure as described in [5].

ANTHOCYANIN QUANTIFICATION

Columbia, AS-*RNS2,* and KO-*rns2* seedlings were grown on +phosphate media and –phosphate media until they were 2 weeks old. Then each line was harvested, frozen in liquid nitrogen, ground up with mortar and pestle, and the powder was poured into a 1% HCl/methanol solution. Samples were then shaken, chloroform was added and samples were centrifuged to separate the organic and aqueous phases. Anthocyanins (in the aqueous phase) were then quantified and calculated as Absorbance = $A_{535} - A_{650}$. [25].

MAGENTA BOXES

Thirty-three magenta boxes were prepared for each media treatment (+phosphate and +RNA/-Pi), which would apportioned to 5 boxes each of Col, WS, and ColxWS plants, and 6 boxes each of *rns1*(ColxWS), *rns2*(ColxWS), and dhm plants, 66 boxes total. The RNA used in the media was from purified torula yeast RNA (see RNase activity gel). Seeds from the 6 lines were sterilized and cold treated, and 5 seeds were placed in each box. The Magenta boxes were kept in the growth chamber under normal growth conditions 16 hour days, 21^{oc} and 69% relative humidity. After 4 weeks, plants were counted every three days to determine the percentage of plants that had bolted, indicated by a stem that was at least 1.25cm tall.

SENESCENCE

Plants for natural senescence experiments were grown on soil for 8 weeks under normal growth conditions. Visual observations were made to determine any differences in rates of natural senescence.

Plants for artificial senescence were grown on soil for 4 weeks under normal growth conditions. Large Petri dishes were lined with moist filter paper in which sample

leaves would be kept during the artificial senescing period. There were 4 Petri dishes used per plant line to view the progression of senescence after 0, 3, 7, and 10 days. 10 leaves per plant line were placed into each Petri dish, thus, 40 leaves from each plant line, Columbia, AS-*RNS2*, and KO-*rns2*, were cut from random plants and placed into the Petri dishes. Each plate was wrapped in foil to keep the leaves in the dark, and kept at room temperature and was not disturbed. After the appropriate amount of days had passed, leaves from each plant line were photographed to record visually their senescing progression.

CHLOROPHYLL QUANTIFICATION

After leaves were photographed for their progression into artificial senescence, they were frozen in liquid nitrogen, ground up, and the powder was poured into 80% acetone. This was allowed to sit at 4^{oC} overnight, centrifuged, and quantified using the following equations:

Chlorophyll *a* (mg/L) = $12.21A_{663} - 2.81A_{646}$

Chlorophyll *b* (mg/L) = $20.13A_{646} - 5.03A_{663}$

Chlorophyll total (mg/L) = $17.3A_{646} + 7.18A_{663}$

The senescence and chlorophyll assays were performed as described by [9].

TuMV INFECTION

Leaf tissue that was previously infected with TuMV-GFP, originally obtained from Steve Whitham's laboratory at ISU, was frozen, ground up, and poured into 20mM sodium phosphate (dibasic) (8vol:1mg virus-leaf-tissue). This was filtered through cheesecloth into a Falcon tube. This solution was rubbed into 3 of the oldest but non-senescing leaves per plant. After 6 days post infection, the plants were taken to a dark

room and a UV lamp was used to view for fluorescent green tissue, indicative of infection [49].

MATERIALS AND METHODS FOR RNS2 LOCALIZATION

CFP-ONLY and CFP-RNS2 CONSTRUCTS

A pBluescript II KS+ plasmid (p1128) that contained *RNS2* cDNA and a pAVA321 plasmid that contained CFP were used to make the CFP-only and CFP-RNS2 constructs. To make CFP-RNS2, CFP was copied via PCR and put into a pGEM-TEasy plasmid to create an easy way to replicate CFP. Using a Quick Site Direct Mutagenesis Kit (Stratagene), the start and stop codons of CFP were altered to other amino acids so that when CFP was inserted between RNS' secretion signal and the rest of RNS2, CFP would not cause transcription errors by having a truncated protein or having the secretion signal missing. When considering base-pair changes, the reading frame was made sure to be kept the same, and these changes included making restriction enzyme sites so that CFP could easily inserted.

AGROBACTERIA *AND* ARABIDOPSIS *TRANSFORMATION*

Agrobacteria were transformed using an electroporation method as described by [23].

Arabidopsis plants were transformed by dipping the inflorescences into the *Agrobacteria*, done according to [10].

PROTOPLAST PREPARTATION

Protoplasts were prepared according procedures described in [37]. The hyperspectral microscope was used at ISU with Robert Doyle's assistance.

CONFOCAL MICROSCOPE CREDITS

Confocal microscope credit must be given to the imaging facility of the Delaware Biotech Institute.

MATERIALS AND METHODS FOR CeRNS

C. elegans N2 nematodes and GFP-expressing nematodes were obtained from the Jo Anne Powell-Coffman lab at ISU. All recipes, procedures and bacterial strains were also obtained from this lab. Recipes, procedures and care of the nematodes were all standard protocols, which includes the Nematode Growth Media (NGM), M9 buffer, OP50 *E.coli* bacteria, N2 *C.elegans,* care of the nematodes, and the bleaching process.

NEMATODE COUNTING PROCESS

Nematodes were viewed under a UV microscope and if they were to be counted for the GFP-RNAi experiments, they were treated with sodium azide to induce paralyzation. Because the GFP expression was not quantified but subjectively observed, nematodes were considered to be “green” if more than 10% of their body was green, otherwise the nematode was considered to be “white”.

CARE OF NEMATODES

Nematodes were always kept at 20^{oC}. OP50 bacteria were spotted onto NGM plates and allowed to grow for 1-2 days before nematodes were placed onto the plate. Nematodes were transferred from plate to plate via the “chunking” method, where a chunk of NGM would be cut out of one Petri dish and turned over onto a new Petri dish.

GFP-RNAi EXPERIMENTS

To start, I bleached the nematodes to begin with a new, uncontaminated population. All nematodes express GFP and look green under a UV microscope. Once a large enough population was reached, the nematodes were separated onto 4 different plates, two of which contained only *unc-22* bacteria and two which contained only CeRNS bacteria, and were allowed to grow for 3 days. This was done to make sure enough nematodes were available for the remainder of the experiment, and to sufficiently knock-out the respective gene. This would also cause the RNAi proteins and enzymes to be indufced to destroy the dsRNA. The nematodes are still observed to be green.

Then one plate of *unc-22* only nematodes were transferred to 5 similar plates, and the other *unc-22* only nematodes were transferred to 5 plates that contained both *unc-22* and GFP RNAi bacteria. The same process was done for the nematodes concerning CeRNS. After 4, 8, 12, and 24 hours a plate of each treatment was taken to count the number of green and “white” nematodes. For ease of counting, the nematodes were treated with sodium azide, which made them permanently paralyzed. The rest of the nematodes were transferred back to plates that contained only the respective RNAi bacteria (unc-22 or CeRNS) without the GFP RNAi bacteria. These nematodes were counted (and paralyzed) every 12 hours for 3 days, in the same way the previous nematodes had been treated, and this was done to determine the “rate of recovery”, where the nematodes could resume normal expression of GFP.

WORKS CITED

1. Ambion, Inc. 2007. The Mechanism of RNA Interference (RNAi). In *Ambion's Online Appendix*, http://www.ambion.com/techlib/append/RNAi_mechanism.html (accessed April 23, 2007).

2. The Arabidopsis Information Resource. In, http://www.arabidopsis.org/ (accessed 4/23, 2007).

3. Bariola, Pauline A., and Pamela J. Green. *Plant ribonucleases.* In*:Ribonucleases: Structures and Functions* (D'Alessio, G. and Riordan, J.F., eds). New York, USA: Academic Press (2007), pp. 163-190.

4. Bariola, Pauline A., Christie J. Howard, Crispin B. Taylor, Michael T. Verburg, Vanita D. Jaglan, and Pamela J. Green. "The Arabidopsis Ribonuclease Gene RNS1 Is Tightly Controlled in Response to Phosphate Limitation." *The Plant Journal* 6, no. 5 (1994): 673-85.

5. Bariola, Pauline A., Gustavo C. MacIntosh, and Pamela J. Green. "Regulation of S-Like Ribonuclease Levels in Arabidopsis. Antisense Inhibition of RNS1 or RNS2 Elevates Anthocyanin Accumulation." *Plant Physiol.* 119, no. 1 (1999): 331-42.

6. Baugh, L. Ryan, Andrew A. Hill, Donna K. Slonim, Eugene L. Brown, and Craig P. Hunter. "Composition and Dynamics of the Caenorhabditis Elegans Early Embryonic Transcriptome." *Development* 130, no. 5 (2003): 889-900.

7. Buchanan, Bob, Wilhelm Gruissem, and Russell Jones. *Biochemistry and Molecular Biology of Plants*. Rockville, Maryland: American Society of Plant Physiologists, 2000.

8. Carter, Clay, Songqin Pan, Jan Zouhar, Emily L. Avila, Thomas Girke, and Natasha V. Raikhel. "The Vegetative Vacuole Proteome of Arabidopsis Thaliana Reveals Predicted and Unexpected Proteins." *Plant Cell* 16, no. 12 (2004): 3285-303.

9. Chapman, D.J. " Qualitative Analysis of Pigments." *In Experimental Phycology: A Laboratory Manual* (1988): pp. 93– 103.

10. Clough, Steve, and Andrew Bent. 1998. Simplified Arabidopsis Transformation Protocol. In, University of Illinois, http://plantpath.wisc.edu/~afb/protocol.html (accessed 4/23, 2007).

11. Diehn, Scott H., Wan-Ling Chiu, E. Jay De Rocher, and Pamela J. Green. "Premature Polyadenylation at Multiple Sites within a Bacillus Thuringiensis Toxin Gene-Coding Region." *Plant Physiol.* 117, no. 4 (1998): 1433-43.

12. Donna, L. Chen, A. Delatorre Carla, Bakker Aleida, and Abel Steffen. "Conditional Identification of Phosphate-Starvation-Response Mutants in Arabidopsis Thaliana." *Planta* 211, no. 1 (2000): 13.

13. Fire, Andrew, SiQun Xu, Mary K. Montgomery, Steven A. Kostas, Samuel E. Driver, and Craig C. Mello. "Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis Elegans." *Nature* 391, no. 6669 (1998): 806.

14. Galili, Gad. "Er-Derived Compartments Are Formed by Highly Regulated Processes and Have Special Functions in Plants." *Plant Physiol.* 136, no. 3 (2004): 3411-13.

15. Gene Summary for Unc-22. In *WormBase*, http://www.wormbase.org/db/gene/gene?name=WBGene00006759;class=Gene (accessed April 23, 2007).

16. Green, P. J. "The Ribonucleases of Higher Plants." *Annual Review of Plant Physiology and Plant Molecular Biology* 45, no. 1 (1994): 421-45.

17. Grishok, Alla, Hiroaki Tabara, and Craig C. Mello. "Genetic Requirements for Inheritance of RNAi in C.Elegans." *Science* 287, no. 5462 (2000): 2494-97.

18. Hara-Nishimura, Ikuko, Ryo Matsushima, Tomoo Shimada, and Mikio Nishimura. "Diversity and Formation of Endoplasmic Reticulum-Derived Compartments in Plants. Are These Compartments Specific to Plant Cells?" *Plant Physiol.* 136, no. 3 (2004): 3435-39.

19. Haseloff, Jim, Kirby R. Siemering, Douglas C. Prasher, and Sarah Hodge. "Removal of a Cryptic Intron and Subcellular Localization of Green Fluorescent Protein Are Required to Mark Transgenic Arabidopsis Plants brightly." *PNAS* 94, no. 6 (1997): 2122-27.

20. Herman, Eliot, and Monica Schmidt. "Endoplasmic Reticulum to Vacuole Trafficking of Endoplasmic Reticulum Bodies Provides an Alternate Pathway for Protein Transfer to the Vacuole." *Plant Physiol.* 136, no. 3 (2004): 3440-46.

21. Hewitt, Matthew M., Jessica M. Carr, Cynthia L. Williamson, and Robert D. Slocum. "Effects of Phosphate Limitation on Expression of Genes Involved in Pyrimidine Synthesis and Salvaging in Arabidopsis." *Plant Physiology and Biochemistry* 43, no. 2 (2005): 91.

22. Irie, Masachika. "Structure-Function Relationships of Acid Ribonucleases: Lysosomal, Vacuolar, and Periplasmic Enzymes." *Pharmacology & Therapeutics* 81, no. 2 (1999): 77.

23. Koncz, Csaba, Kinga Németh, George P. Rédei, and Jeff Schell. "T-DNA Insertional Mutagenesis in Arabidopsis." *Plant Molecular Biology* Volume 20, no. Number 5 (1992): 963-76.

24. Koornneef, Maarten, Carlos Alonso-Blanco, Anton J. M. Peeters, and Wim Soppe. "Genetic Control of Flowering Time in Arabidopsis." *Annual Review of Plant Physiology and Plant Molecular Biology* 49, no. 1 (1998): 345-70.

25. Lange, H., W. Shropshire, Jr., and H. Mohr. "An Analysis of Phytochrome-Mediated Anthocyanin Synthesis." *Plant Physiol.* 47, no. 5 (1971): 649-55.

26. LeBrasseur, Nicole D. "Regulation and Function of *Arabidopsis Thaliana* Secreted Ribonucleases." *PhD Thesis* (2001).

27. LeBrasseur, Nicole D., Gustavo C. MacIntosh, Miguel A. Perez-Amador, Maki Saitoh, and Pamela J. Green. "Local and Systemic Wound-Induction of RNase and Nuclease Activities in Arabidopsis: RNS1 as a Marker for a JA-Independent Systemic Signaling Pathway." *The Plant Journal* 29, no. 4 (2002): 393-403.

28. Lehmann, Karin, Bettina Hause, Dorit Altmann, and Margret Kock. "Tomato Ribonuclease LX with the Functional Endoplasmic Reticulum Retention Motif HDEF Is Expressed During Programmed Cell Death Processes, Including Xylem Differentiation, Germination, and Senescence." *Plant Physiol.* 127, no. 2 (2001): 436-49.

29. Lers, Amnon, Lilian Sonego, Pamela J. Green, and Shaul Burd. "Suppression of LX Ribonuclease in Tomato Results in a Delay of Leaf Senescence and Abscission." *Plant Physiol.* 142, no. 2 (2006): 710-21.

30. Levy, Yaron Y., and Caroline Dean. "The Transition to Flowering." *Plant Cell* 10, no. 12 (1998): 1973-90.

31. MacIntosh, G. C., R. M. Ulloa, M. Raices, and M. T. Tellez-Inon. "Changes in Calcium-Dependent Protein Kinase Activity During in Vitro Tuberization in Potato." *Plant Physiol.* 112, no. 4 (1996): 1541-50.

32. MacIntosh, Gustavo C., Pauline A. Bariola, Ed Newbigin, and Pamela J. Green. "Characterization of Rny1, the Saccharomyces Cerevisiae Member of the T2 RNase Family of Rnases: Unexpected Functions for Ancient Enzymes?" *PNAS* 98, no. 3 (2001): 1018-23.

33. Matsushima, Ryo, Yasuko Hayashi, Maki Kondo, Tomoo Shimada, Mikio Nishimura, and Ikuko Hara-Nishimura. "An Endoplasmic Reticulum-Derived Structure That Is Induced under Stress Conditions in Arabidopsis." *Plant Physiol.* 130, no. 4 (2002): 1807-14.

34. Muller, Renate, Lena Nilsson, Christian Krintel, and Tom Hamborg Nielsen. "Gene Expression During Recovery from Phosphate Starvation in Roots and Shoots of Arabidopsis Thaliana." *Physiologia Plantarum* 122, no. 2 (2004): 233-43.

35. Richman, A. D., W. Broothaerts, and J. R. Kohn. "Self-Incompatibility Rnases from Three Plant Families: Homology or Convergence?" *Am. J. Bot.* 84, no. 7 (1997): 912-.

36. Sanchez-Calderon, Lenin, Jose Lopez-Bucio, Alejandra Chacon-Lopez, Alfredo Cruz-Ramirez, Fernanda Nieto-Jacobo, Joseph G. Dubrovsky, and Luis Herrera-Estrella. "Phosphate Starvation Induces a Determinate Developmental Program in the Roots of Arabidopsis Thaliana." *Plant Cell Physiol.* 46, no. 1 (2005): 174-84.

37. Sheen, J. 2002. A Transient Expression Assay Using Arabidopsis Mesophyll Protoplasts. In, http://genetics.mgh.harvard.edu/sheenweb/ (accessed 4/23, 2007).

38. Signal "T-DNA Express" Arabidopsis Gene Mapping Tool. 2007. In, http://signal.salk.edu/cgi-bin/tdnaexpress (accessed 4/23, 2007).

39. Simpson, Gordon G., and Caroline Dean. "Arabidopsis, the Rosetta Stone of Flowering Time?" *Science* 296, no. 5566 (2002): 285-89.

40. Song, Liang, Meng-Hsuan Han, Joanna Lesicka, and Nina Fedoroff. "Arabidopsis Primary Microrna Processing Proteins Hyl1 and Dcl1 Define a Nuclear Body Distinct from the Cajal Body." *PNAS* 104, no. 13 (2007): 5437-42.

41. Taylor, C. B., P. A. Bariola, S. B. DelCardayre, R. T. Raines, and P. J. Green. "RNS2: A Senescence-Associated RNase of Arabidopsis That Diverged from the S-Rnases before Speciation." *PNAS* 90, no. 11 (1993): 5118-22.

42. Ticconi, Carla A., Carla A. Delatorre, and Steffen Abel. "Attenuation of Phosphate Starvation Responses by Phosphite in Arabidopsis." *Plant Physiol.* 127, no. 3 (2001): 963-72.

43. van Tienderen, Peter H., Ibtisam Hammad, and Frits C. Zwaal. "Pleiotropic Effects of Flowering Time Genes in the Annual Crucifer *Arabidopsis Thaliana* (Brassicaceae)." *American Journal of Botany* 83, no. 2 (1996): 169-74.

44. von Arnim, A. G., X. W. Deng, and M. G. Stacey. "Cloning Vectors for the Expression of Green Fluorescent Protein Fusion Proteins in Transgenic Plants." *Gene* 221, no. 1 (1998): 35.

45. Walsh, John A., and Carol E. Jenner. "Turnip Mosaic Virus and the Quest for Durable Resistance." *Molecular Plant Pathology* 3, no. 5 (2002): 289-300.

46. Waterhouse, Peter M., Ming-Bo Wang, and Tony Lough. "Gene Silencing as an Adaptive Defence against Viruses." *Nature* 411, no. 6839 (2001): 834.

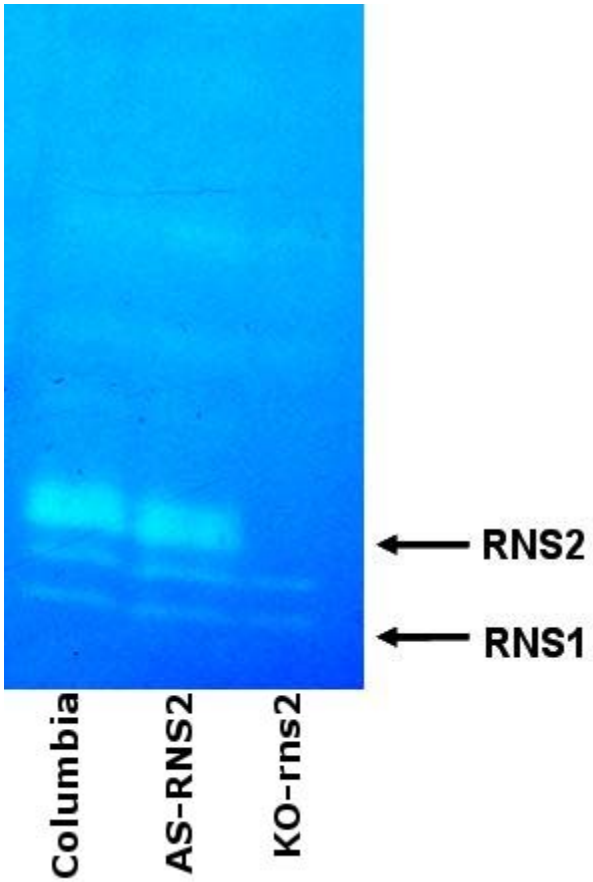
47. Williamson, Lisa C., Sebastien P. C. P. Ribrioux, Alastair H. Fitter, and H. M. Ottoline Leyser. "Phosphate Availability Regulates Root System Architecture in Arabidopsis." *Plant Physiol.* 126, no. 2 (2001): 875-82.

48. Xiong, Yan, Anthony L. Contento, and Diane C. Bassham. "Atatg18a Is Required for the Formation of Autophagosomes During Nutrient Stress and Senescence in Arabidopsis Thaliana." *The Plant Journal* 42, no. 4 (2005): 535-46.

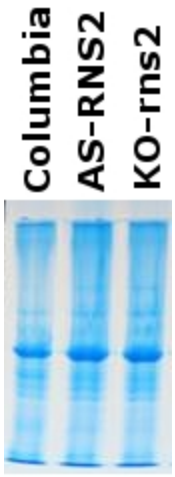
49. Yang, Chunling, Rong Guo, Fei Jie, Dan Nettleton, Jiqing Peng, Tyrell Carr, Joanne M. Yeakley, Jian-Bing Fan, and Steven A. Whitham. "Spatial Analysis of *Arabidopsis Thaliana* Gene Expression in Response to *Turnip Mosaic Virus* Infection." *The American Phytopathological Society* 20, no. 4 (2007): 358-70.

50. Yen, Yang, and Pamela J. Green. "Identification and Properties of the Major Ribonucleases of Arabidopsis Thaliana." *Plant Physiol.* 97, no. 4 (1991): 1487-93.

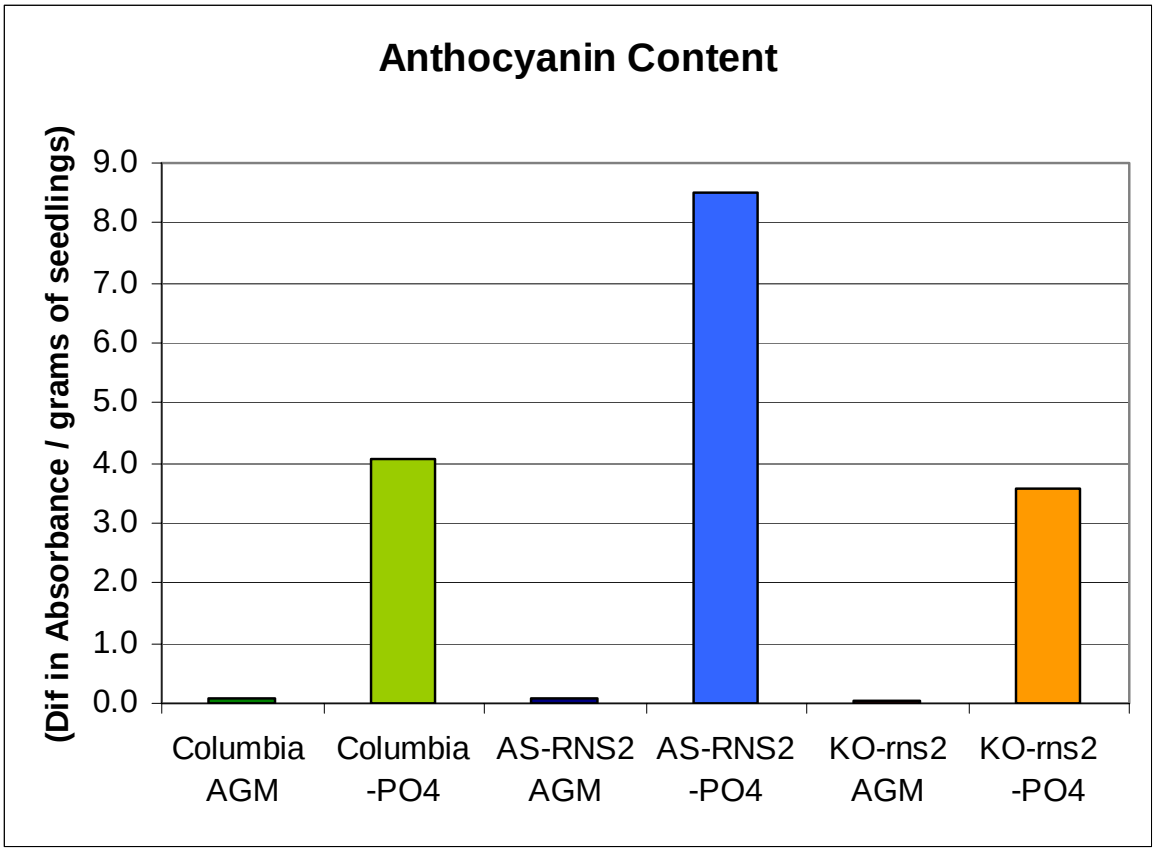
FIGURES



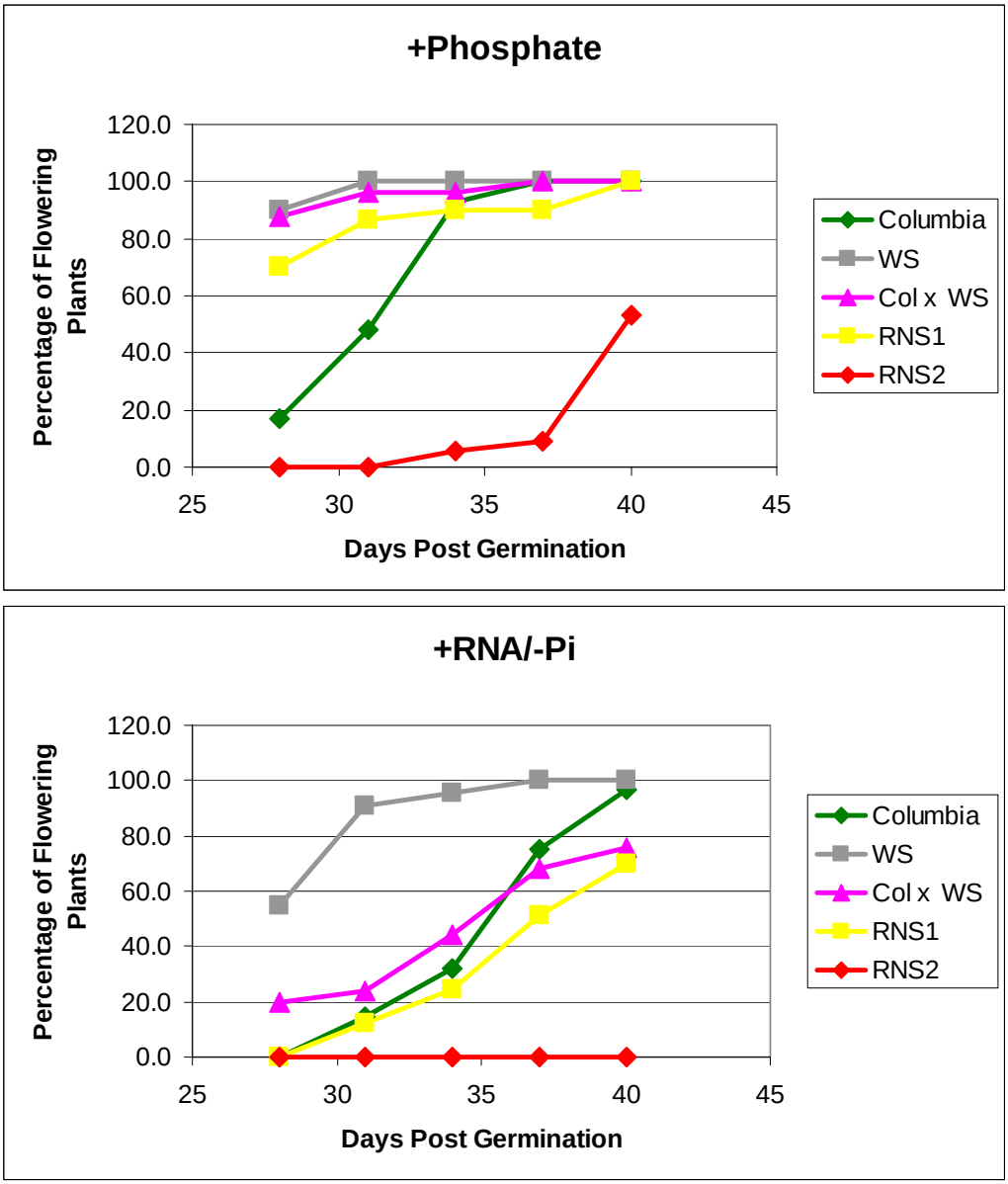
FIGURE#1: Determination of RNS2 activity in an RNase activity gel. The KO-*rns2* mutant was confirmed, and the band which is associated with RNS2 was discovered. Proteins were extracted from 4-week-old plants, and 40ug were analyzed in an RNase activity gel. Columbia: wild-type; AS-*RNS2*: antisense *RNS2*; KO-*rns2*: knock-out *rns2* mutant. The position of RNS1 was previously known and it is shown as a reference.



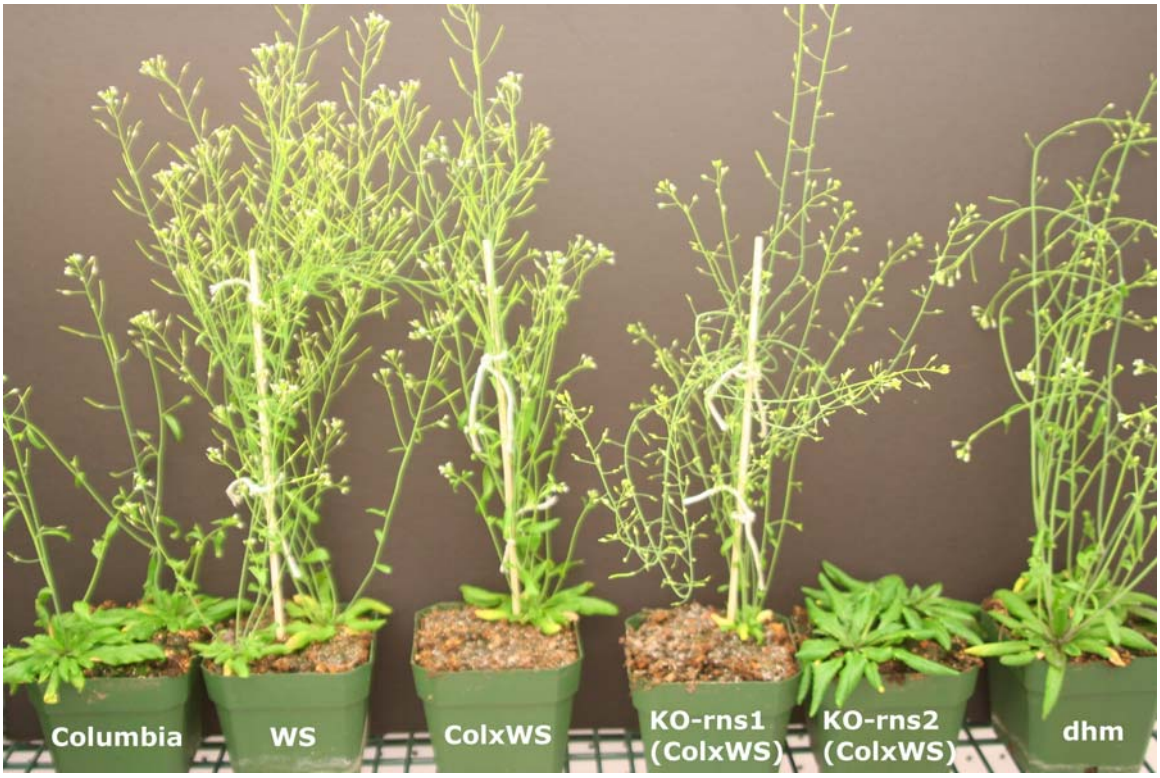
FIGURE#2: Coomassie gel verification of equal protein loading for RNase activity gel in FIGURE1. 10ug of protein used in the RNase activity gel were analyzed to make sure the differences in RNase activity were not due to unequal protein loading. Equal loading was verified using a Coomassie blue stain. Columbia: wild-type; AS-*RNS2*: antisense *RNS2*; KO-*rns2*: knock-out *rns2* mutant.



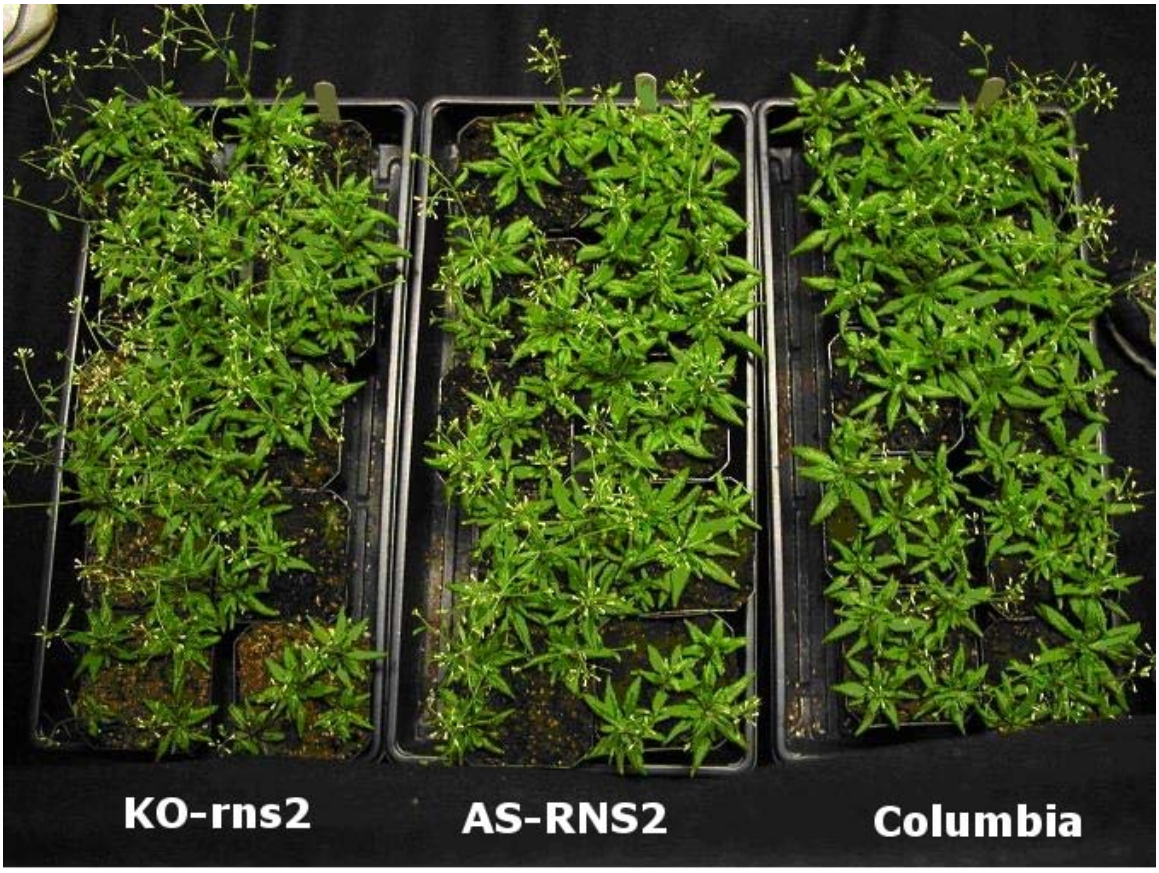
FIGURE#3: Anthocyanin content of plants grown on +phosphate and –phosphate growth media. Anthocyanin content was analyzed from Columbia, AS-*RNS2*, and KO-*rns2* plants that were grown on either +phosphate or –phosphate media. Almost no anthocyanins had accumulated in plants grown on +phosphate media, but all 3 lines of plants showed an increase in anthocyanin content when grown on –phosphate media. Compared to Columbia (wild-type), the KO-*rns2* anthocyanin content was not significantly different, but the AS-*RNS2* line had twice the amount of anthocyanins. There are no error bars because this experiment was performed only once; more replicates would be necessary for statistical analysis.



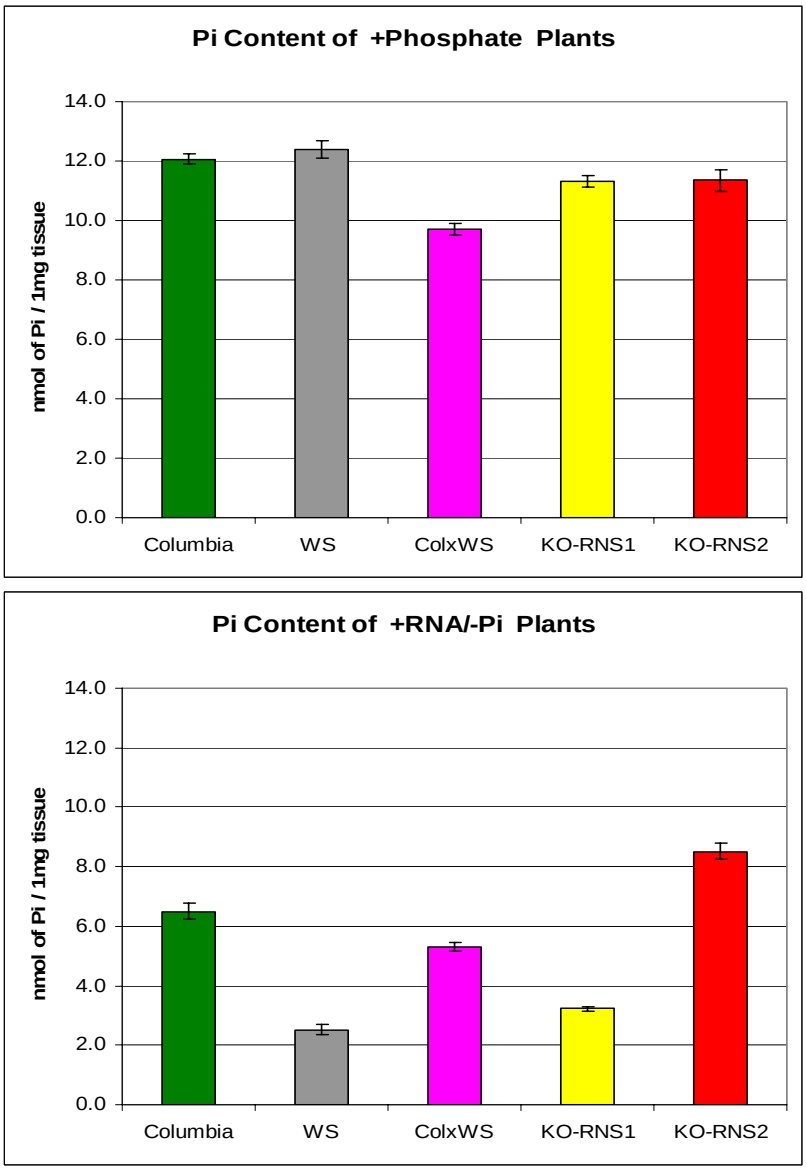
FIGURE#4: The percentage of flowering plants when grown on +phosphate or +RNA/-Pi growth media. Columbia, WS, ColxWS, KO-*rns1*(ColxWS), and KO-*rns2*(ColxWS) plants were grown in Magenta boxes that contained either +phosphate or +RNA/-Pi media. When the plants were 4 weeks old they were counted every three days to determine the percentage that had flowered. A plant was counted as having flowered if its stem was at least 1.25cm tall. When grown on +phosphate media, only KO-*rns2* plants show a delay in flowering. All plants grown on +RNA/-Pi media show a delay in flowering, but eventually do flower, except the KO-*rns2* plants, which do not flower even after almost 6 weeks.



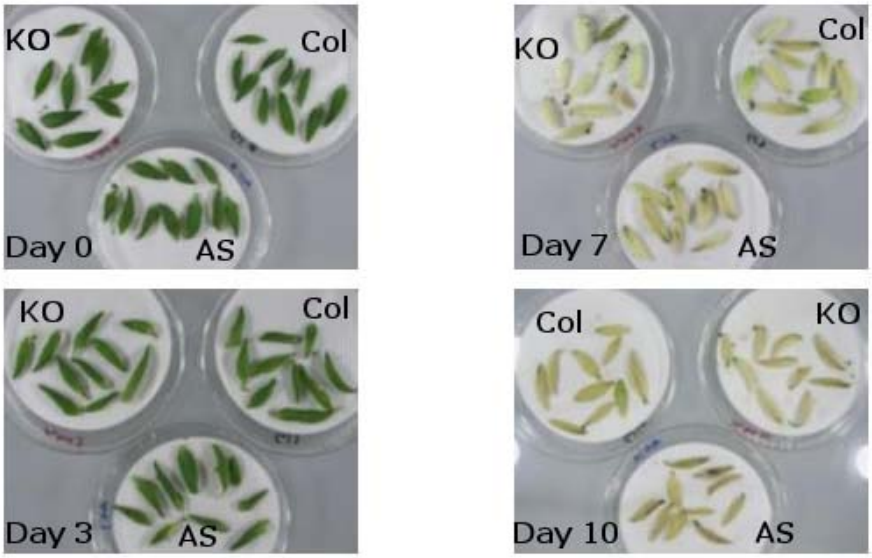
FIGURE#5: 6 lines of plants grown on soil for 4 weeks. The plants shown here are: (left to right) Columbia, WS, ColxWS, KO-*rns1*(ColxWS), KO-*rns2*(ColxWS), dhm. As seen when grown on +phosphate media, only the KO-*rns2*(ColxWS) plants show a delay in flowering when compared all lines of plants, and especially compared to its wild-type, ColxWS.

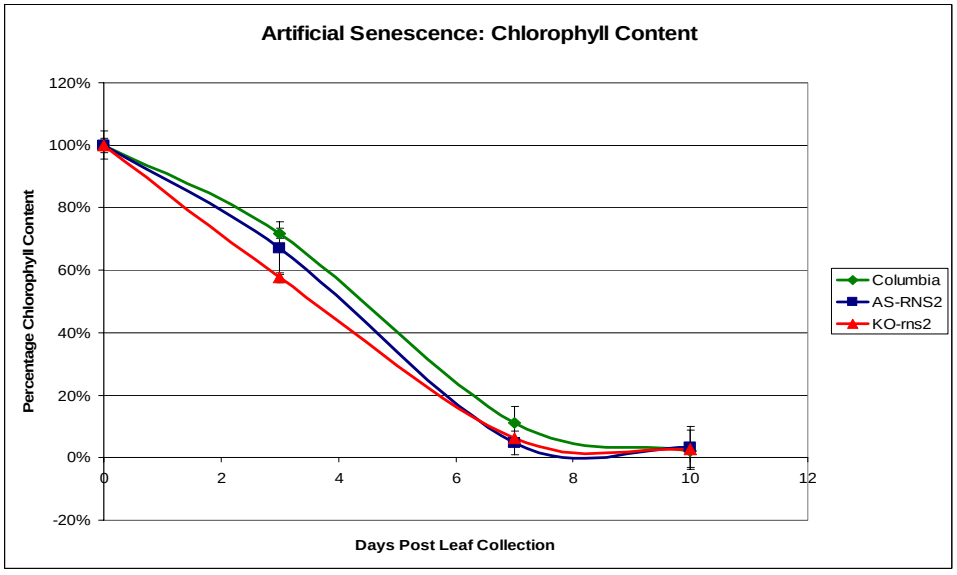


FIGURE#6: Wild-type, AS-*RNS2*, and KO-*rns2* plants grown on soil for 4 weeks. When grown on soil, there are no flowering time differences seen among these plant lines.

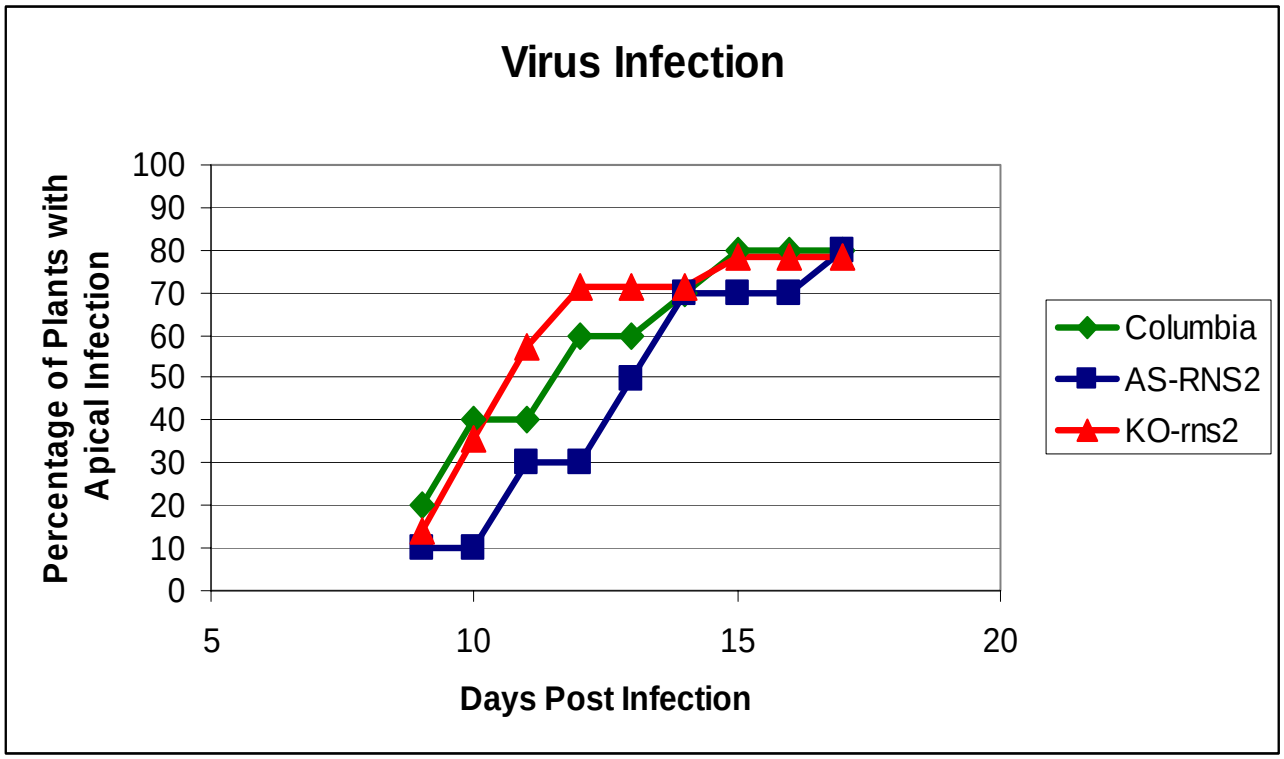


FIGURE#7: Inorganic phosphate content of the 5 lines of plants grown on +phosphate or +RNA/-Pi media. All lines of plants grown on +phosphate media contained approximately the same amount of Pi. However, when grown on +RNA/-Pi media, all lines of plants contained about half the amount of Pi. ColxWS is the wild-type control for KO-*rns1*(ColxWS) and KO-*rns2*(ColxWS). When compared to its wild-type, KO-*rns1*(ColxWS) contained about half the amount of Pi, and KO-*rns2*(ColxWS) contained twice the amount of Pi. This data is taken from one experimental trial, but the samples were broken down into three sub-samples, and the variance between the sub-samples is indicated by the error bars.

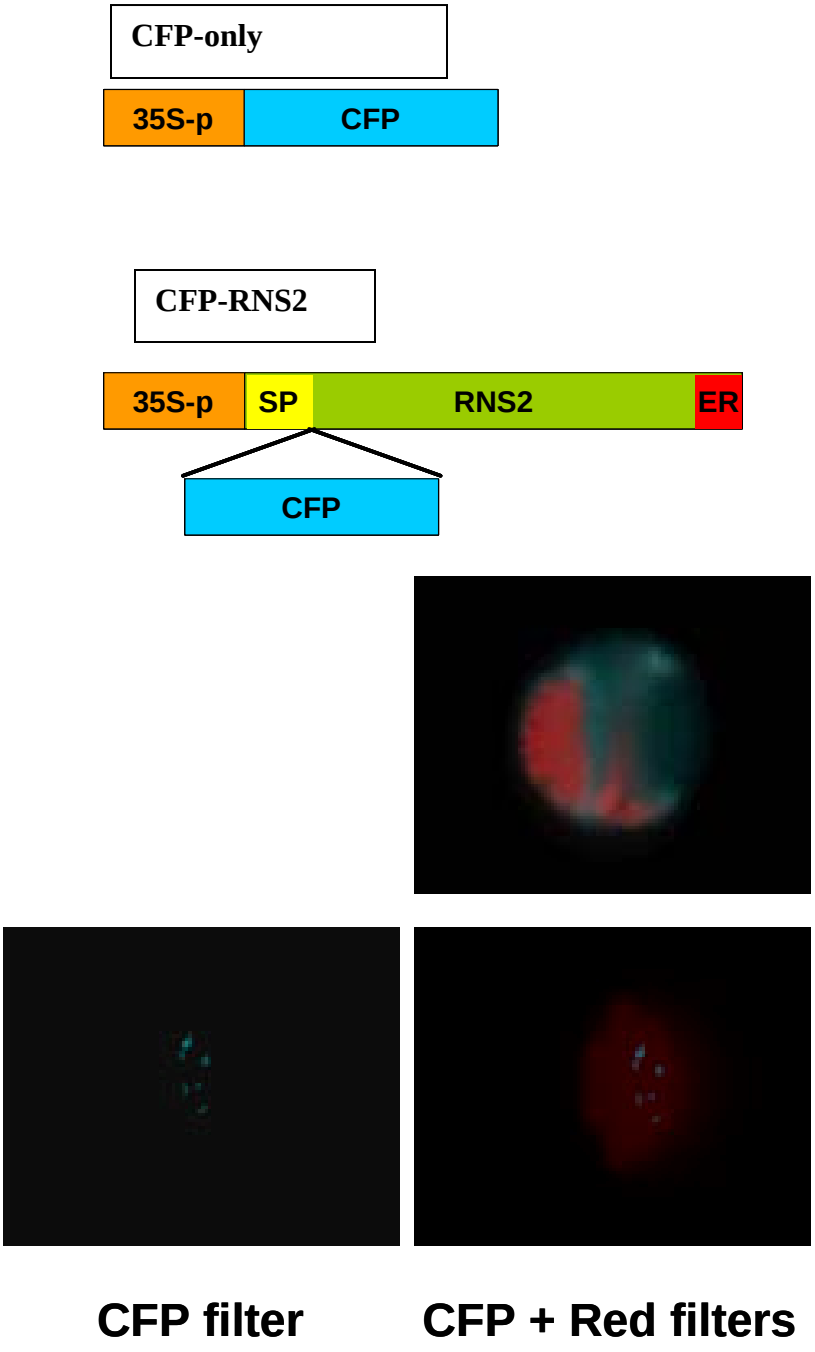




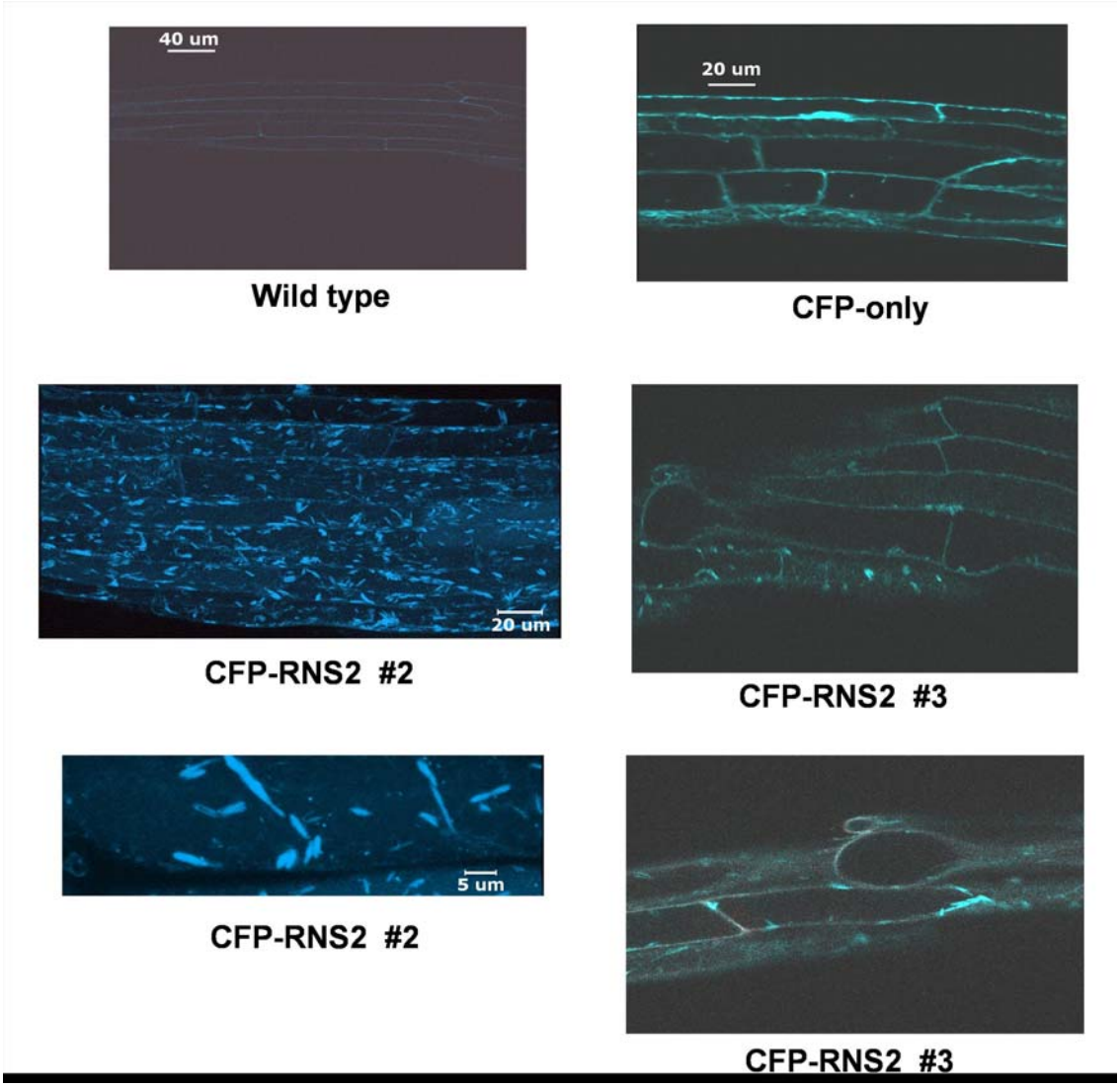
FIGURE#8: Artificial senescence and chlorophyll content of Columbia, AS-*RNS2*, and KO-*rns2* plants. Photographs were taken of artificially senescing leaves to visually document the progression of senescence and chlorophyll degradation. The chlorophyll content of artificially senescing leaves was quantified from Columbia, AS-*RNS2*, and KO-*rns2* plants. The photos and graph show the data collected from one independent trial and are representative of the 4 independent trials conducted for artificial senescence. Chlorophyll content declines in all the plant lines, but the differences are not significant. Col: Columbia; AS: AS-*RNS2*; KO: KO-*rns2*. This experiment was performed once, but each sample was separated into three sub-samples. The error bars indicate the variance between the three sub-samples.



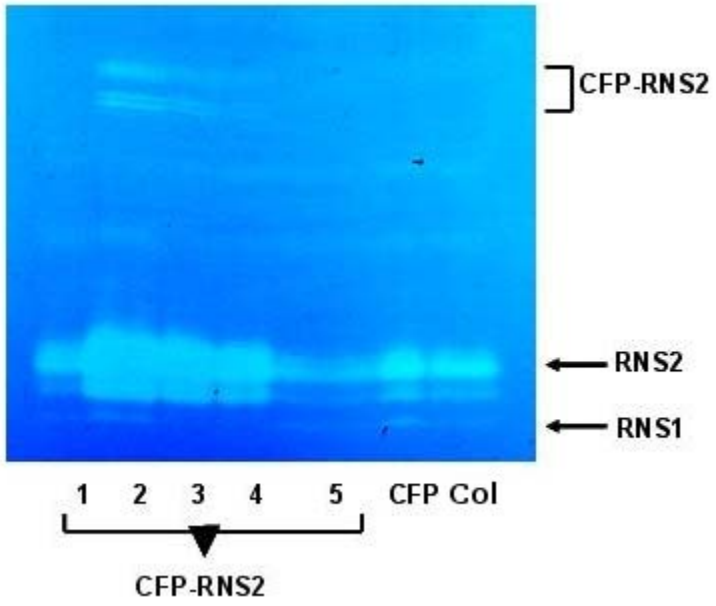
FIGURE#9: Percentage of infected apical tissue in *Arabidopsis* Columbia, AS-*RNS2*, and KO-*rns2* plants. In each plant, 3 of the oldest, non-senescing rosette leaves were infected with TuMV-GFP, and after 6 days were viewed under UV light everyday for evidence of apical infection. A plant was considered to have apical infection if any tissue in the top 5cm was viewed to be fluorescent green under UV light. There were no significant differences found between rates of infection or percentage infection among the 3 lines of plants. There are no error bars because this experiment was performed once.



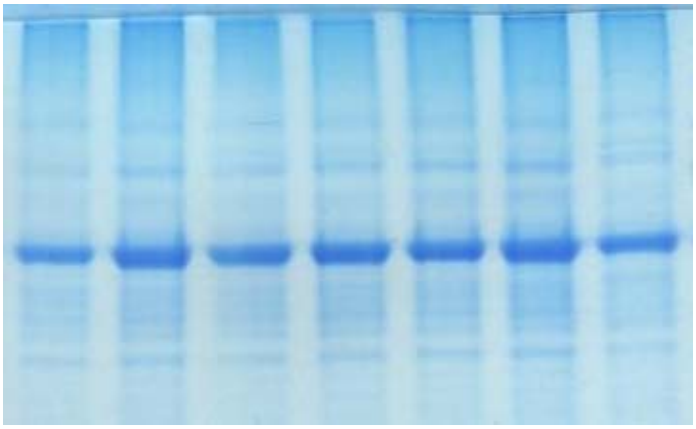
FIGURE#10: CFP and CFP-RNS2 constructs, and verification of CFP expression in protoplasts. The top picture shows the 2 constructs used in creating CFP-only and CFP-RNS2 transgenic plants. The bottom picture shows hyperspectral microscope images of protoplasts prepared from transgenic CFP-RNS2 plants. The CFP is being localized to particulate structures that are not chloroplasts. These preliminary images confirm the expression of CFP and possible localization of RNS2.



FIGURE#11: Confocal microscope images of CFP expression within CFP-RNS2 root tissue and wild-type root tissue. The wild-type image only shows autofluorescence. CFP-only shows CFP expression within the cytoplasm. In both CFP-RNS2 #2 and #3 lines, CFP is being localized to ER-bodies.



FIGURE#12: CFP-RNS2 maintains RNase activity. Also, an increase in RNase activity from lines #2, #3, and #4 compared to the wild-type can be observed. Protein samples were obtained and analyzed as described in Figure1.



1 2 3 4 5 CFP COL

FIGURE#13: Verification of equal protein loading for FIGURE#12. 10ug of the protein used in the RNase activity gel were analyzed to make sure the differences in RNase activity were not due to unequal protein loading. Equal loading was verified using a Coomassie blue stain. Lanes 1-5 correspond to RNS2-CFP samples #1-5, and the CFP and COL lanes correspond to CFP-only and wild-type Columbia, respectively.

atgaaacttctccttcttctctgcatatcctgtataccccctagcctattct**catgatggtgagccatttga**
ctacctaatgttcaccacaatctacccaaccgccgtctgccgtgccgatgatgattcagtccctgaatcct
gtgagattccaagtggtacccctcaatggtcaattcacggattgtggccaaactttgagaatggctcgtac
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ggacatgtgctcaaagtgagaagctcttcgagtcagagttggcttacttcacagaggtaatgaaagtcttc
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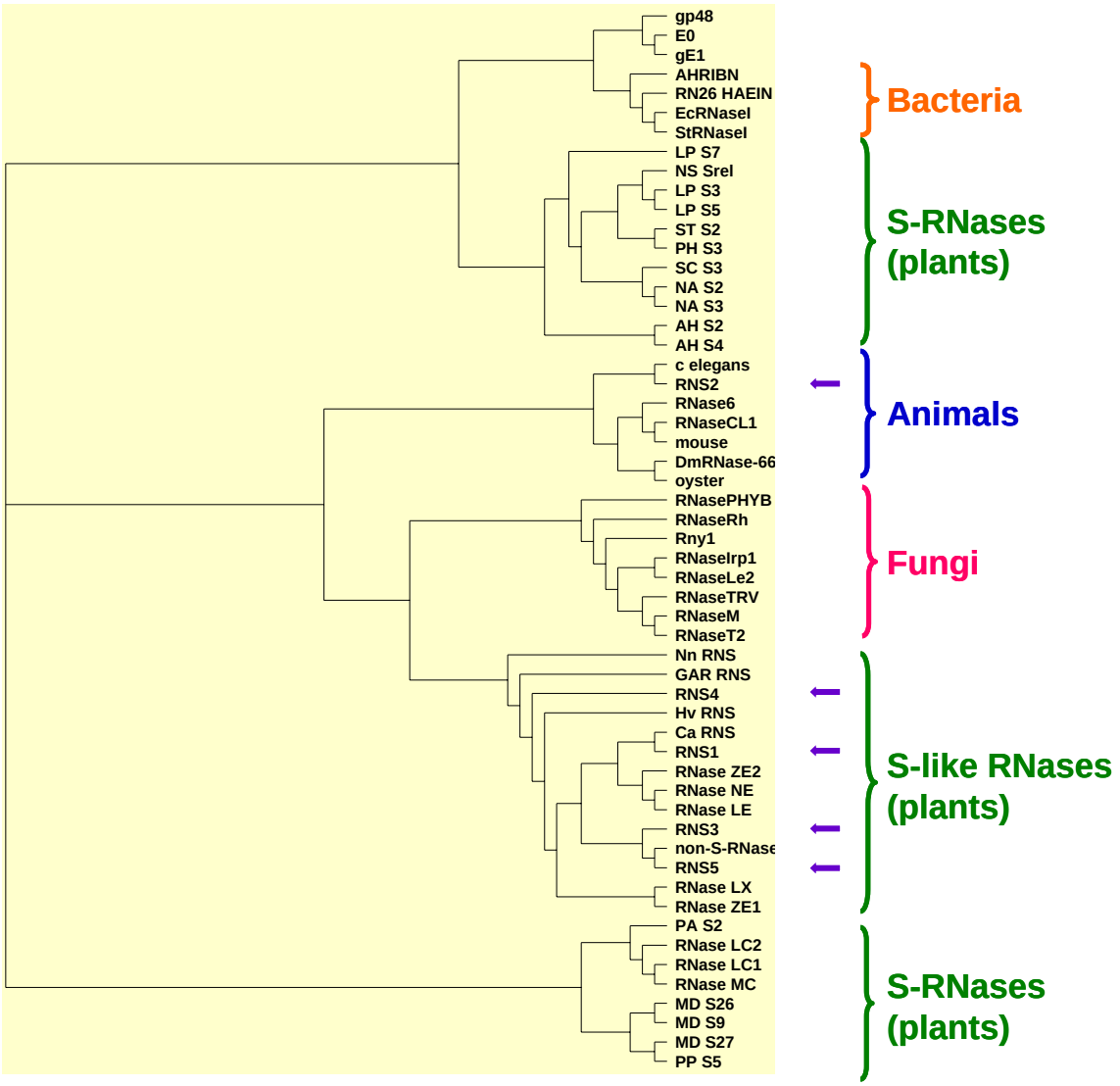
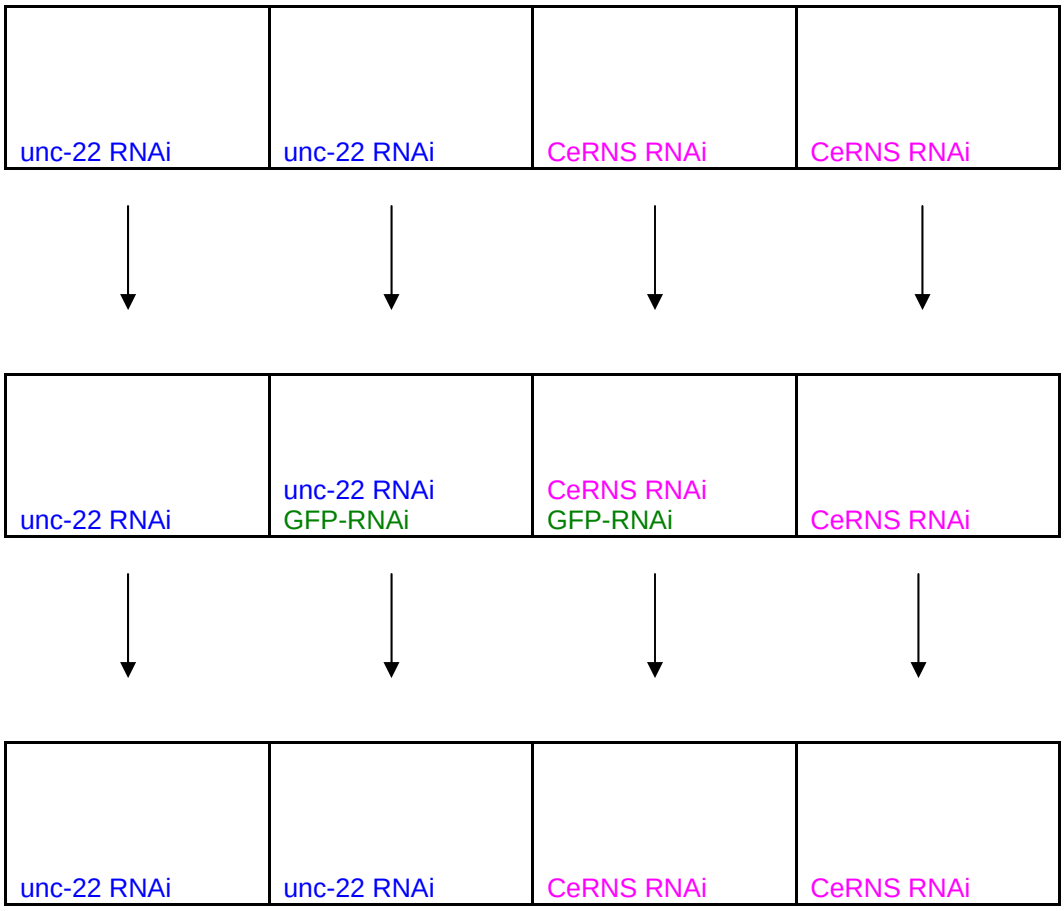
ccacatcgccatctattttcggacgaatctggagcgctatcaaaaatattggaaattaa

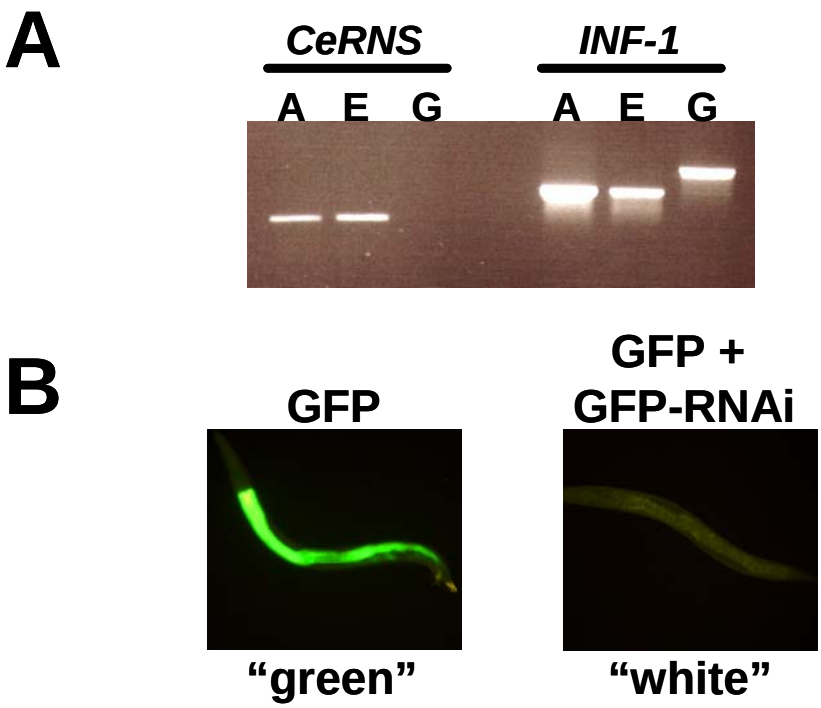
FIGURE#14: The cDNA sequence of *CeRNS* (K10C9.3). 5 exons make up the cDNA for the *CeRNS* gene in *C.elegans.* Primers that were used to detect the presence of CeRNS are in bold and underlined.



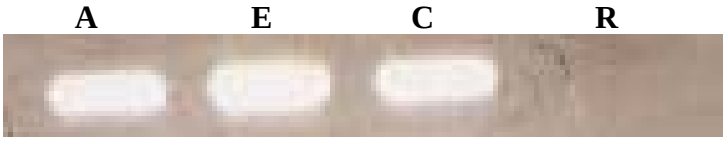
FIGURE#15: Protein phylogenetic tree of T2 RNases. This diagram shows the similarity between amino acid sequences of T2 RNases, and how families appear in clusters, such as the S- and S-Like RNases. Arrows notate where the 5 *Arabidopsis thaliana* T2 S-Like RNases are located. Note that the amino acid sequence of *RNS2* is more similar to *C.elegans'* RNase than to any of *Arabidopsis'* other 4 S-Like RNases. [Phylogenetic tree taken from Gustavo MacIntosh, unpublished.]



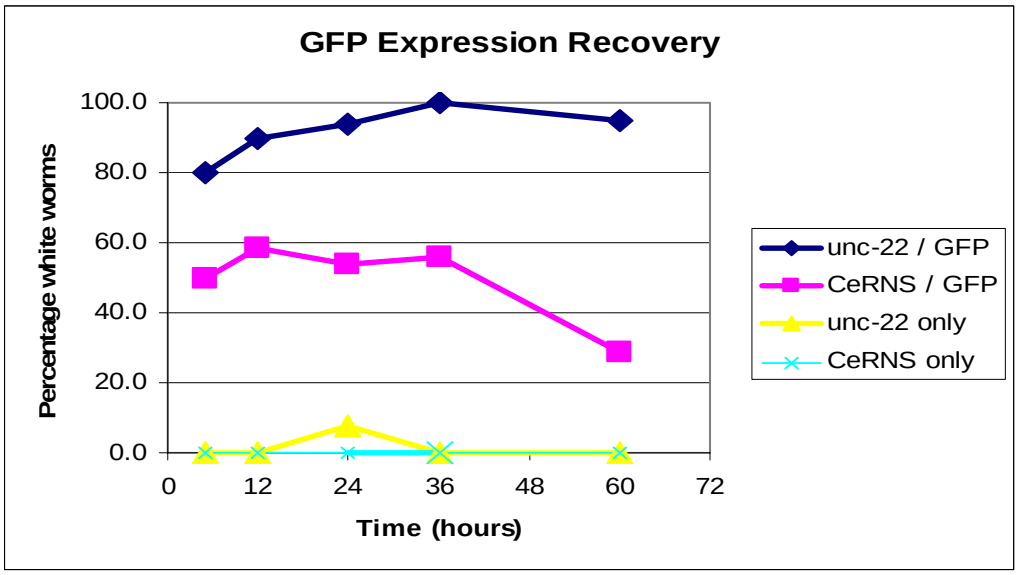
FIGURE#16: Schematic of *C.elegans* RNAi experiments. Each box represents a Petri dish that contains the type(s) of RNAi inducing bacteria on each plate. The nematodes are transferred to each plate via the "chunking" method. Nematodes are transferred from their starting plate (row1) to the next plate (row2) after 3 days, after a large enough population had been acquired. Then nematodes are transferred again (row3) after 24 hours. These different bacterial treatments would determine if CeRNS affects RNAi rates in *C.elegans.*



FIGURE#17: PCR verification of *CeRNS* expression, and verification of GFP-RNAi in *C.elegans.* A: adult cDNA; E: embryo cDNA; G: genomic DNA. Panel A: *CeRNS* primers are used in PCR to determine if *CeRNS* is being expressed in *C.elegans*. INF1 primers are used as a control. A band is not seen in the genomic *CeRNS* lane because the amplified region was too large for PCR. Panel B: Shows UV microscope images of "green" (GFP-expressing) and "white" (GFP-RNAi) nematodes.



FIGURE#18: *CeRNS* is expressed in embryos of *C.elegans* and CeRNS RNAi eliminates *CeRNS* expression. A: adult; E: embryo; C: control; R: CeRNS RNAi. For samples A and E, RNA was collected and then reverse-transcribed from normal, non-GFP expressing nematodes. For samples C and R, RNA was collected then reverse-transcribed from GFP-expressing nematodes. Samples A, E, and C were fed normal bacteria. Sample R was fed CeRNS-RNAi bacteria. For all 4 samples, PCR was conducted to detect the presence of CeRNS cDNA to determine if RNAi was silencing expression of CeRNS.



FIGURE#19: Representative data collected for *CeRNS* RNAi experiments. Because there is no significant difference or consistency between the 2 experimental treatments, it cannot be concluded that *CeRNS* affects RNAi rates in *C.elegans*.