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Biology 240W; Section 029

3/22/2012

The Relationship Between low Levels of Phosphate and Sulfur and There Affect on *Arabidopsis thaliana* microRNA Expression

Introduction:

The first land plants arose in the Paleozoic period, about 420 million years ago. Initially heterotrophic organisms that eventually evolved into different forms of algae, specifically green algae, ate cyanobacteria, which were early photosynthetic organisms that were polluting the environment with oxygen. Out of those algae, Charophyte green algae evolved to eventually form the lush forests that we can see today (Basal Plants, Dr. Axtell's lecture 2012). The plant that is examined in more detail in this lab is the *Arabidopsis thaliana,* out of the lush forests this plant was picked because it is a model organism in used often in plant biology. The *Arabidopsis* is a member of the *Brassicaceae* family or more commonly called the mustard family, this family includes other plants like cabbage and radishes. *Arabidopsis* is known as a model plant for various reasons, one reason being this plant has the ability to go from a seed to maturation within a few weeks and since the *Arabidopsis* can easily grow in restricted areas this plant is perfect for laboratory settings (Collins, n.d.). All five of its chromosome has been extensively mapped and the *Arabidopsis* genome has been almost entirely sequenced, 115.4 megabases of a total genome of 125 megabases (Nature 408, 2000). All these reasons contribute to why this plant is seen in almost all plant labs around the world.

Most modern plants are photoautotrophic, like their algae ancestors, this means that most plants must go through photosynthesis to gain sugars and other necessary nutrients. All the nutrients received through those various forms of synthesis are synthesized from inorganic molecules, making plants metabolically complete. The major nutrients that are required by plants are known as macronutrients, contrary to micronutrients which are nutrients that are still required by the plant, but only in very small quantities. The two

most important macronutrients, which make up 90% of the plant, are carbon and oxygen. The two macronutrients important to this lab are sulfur and phosphorus. Sulfur is picked up from sulfate ions and phosphorus from phosphate ions. Sulfur is a component required for the synthesis of proteins and coenzymes. Phosphorus on the other hand is an element of nucleic acids, phosolipids, ATP and like sulfur is important for coenzymes. In nature, phosphorus and sulfur are limiting for plant growth because they must be scavenged from the soil and not all soil as ample supplies. Phosphate for example, instead of leaching out of the soil when it rains phosphate forms insoluble compounds with various substances that cannot be taken up by the plant. When there are low levels of phosphate, more branching occurs in the roots, this is because the plant has to extend out more roots in search of more phosphate. By having to create more roots, the plant wastes crucial energy needed to grow (Plant Nutrition, Dr. Axtell's lecture 2012). Sulfur is an important nutrient because sulfur is an essential part of a plant's growth. Sulfur in plants is also highly linked with nitrogen obtainability and growth rate (Hawkesford, Malcolm and Kok). This is why fertilizer plays a key role in growing healthy plants because the fertilizer contains these limited nutrients in available forms (Plant Nutrition, Dr. Axtell's lecture 2012).

microRNA is a 22 nucleotide, post-transcriptional regulator. Unlike mRNA, miRNA are not translated into proteins, instead they are single-stranded, short regulators that search for target mRNAs. miRNAs cause the degradation of mRNAs to increase and lower the rate of mRNA translation, this is known as negative post-transcriptional control. miRNA are able to seek out and destroy the target mRNA by hooking onto proteins that allow them to function and attach to the mRNA, through this process the miRNA stops and regulates gene expression (Axtell, Burpee and Nelson 2012). When a plant undergoes

nutrient stresses, the plants miRNA regulate adaptive responses. Normally miRNAs lower the abundance of their specific target mRNAs using post-transcriptional regulation, but in the case of sulfate and phosphate stress some miRNA up-regulate, specifically miR395 regulates when there is a sulfate deficiency and miR399 when there is a phosphate deficiency (Chiou, 2007). Another type of miRNA is miR398, which highly down regulates CSD when there is a phosphate limitation. miR398 also acts the same way when there is a Carbon or Nitrogen deficiency (Zhu, 2010). This knowledge can be related back to the prior fact that sulfur and nitrogen are linked, meaning miR398 also shares the same relationship with sulfur as it does nitrogen (Hawkesford, Malcolm and Kok). The final miRNA that was observed in this lab is miR156, which up regulates SPL genes. SPL genes code for proteins that are involved in DNA binding as well as various larger proteins. Since as stated earlier phosphorus is involved with nucleic acids and sulfur is involved in proteins, lowering these would cause an increase in miR156 (Wang, 2009).

The goal of this lab is to determine how the amounts of various forms of *Arabidopsis thaliana* miRNA are affected when the plant is grown in either a low phosphorus media or a low sulfur media. Some questions that will be addressed in this lab are the how will the *Arabidopsis* be affected in a low phosphate media? How will the plant be affected if there is no sulfur in the media? Also in which media do the various miRNAs have high concentrations compared to lower concentrations? From the research gathered, I predict there will be a higher concentration of miR395 in the no sulfur media and a higher concentration of miR399 in the low phosphate media.

Materials and Methods:

This lab required various types of equipment and materials. In week one each group used one of three types of sterile media plates, which were petri dishes with agars of different nutrient levels, to plant and grow *Arabidopsis* seedlings. The three media plates were full media, low phosphate and no sulfur and there was an equal representation of each of these plate types in the lab. About three weeks were allowed to pass in order for the plants to germinate and grow to a substantial size before any extractions took place. The extraction of small RNA was done using the Sigma mirPremier microRNA isolation kit with an established protocol (Axtell, Burpee and Nelson 2012).

The cDNA for all three media types that the above protocol created was subjected then to qPT-PCR. To prepare the samples, the four microRNAs mentioned in the intro; miR156, miR395, miR398 and miR399 were inserted as miRNA primers into the cDNA from each plate type. A control of U6 primer was also inserted into each of the plate types cDNA to establish a control. A no-template control, which was simple water, was also used and if any DNA amplification occurred in this sample it meant that there must have been some sort of contamination. Specifically I used RNA extracted from a low phosphate plate and used the miRNA primer 398, in order to gain data on how a low phosphate environment affects the miR398 in the plant. The purpose for using any type of PCR is to amplify the amount of DNA present to in order to allow for easier detection, but PCR only works for DNA so the RNA collected was put through reverse transcription. qRT-PCR or qPCR is used to measure gene expression and can measure the expression of miRNAs. qPCR is a way of detecting and tracing the amplification of DNA. It does this by using fluorescent dyes that bind to double stranded DNA only and then monitors that are capable

of detecting this fluorescent dye. The amount of amplification is then tracked and displayed as a graph that can then be interpreted (Axtell, Burpee and Nelson 2012).

Specific formulas and charts were used with the data retrieved from the qPCR to retrieve the final bar graphs displayed in the results section. Figure 1 shows a fluorescence vs. time graph, the intersections on the threshold line are known as cycle thresholds. The lower the cycle thresholds the higher the concentration of target DNA. In a perfect world the DNA should double every cycle, but this just isn't true. By diluting the

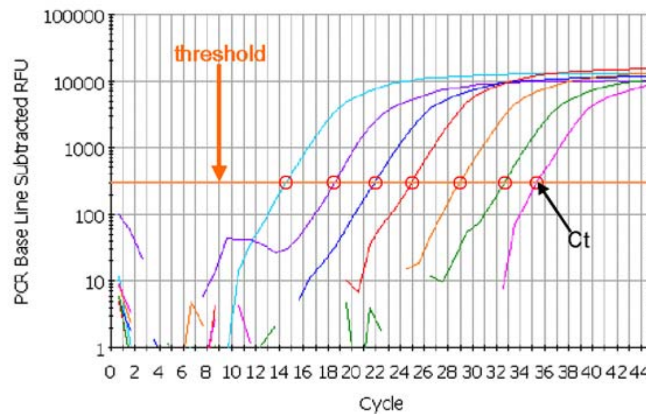

Figure 1. Fluorescence vs. Time Graph taken off the lab manual.

target cDNA and graphing the results in a cycle threshold vs. log10 dilution graph, the resulting graph will show a linear relationship. Figure 2 shows a sample chart taken and Figure 3 shows the graph produced from that chart. The slope of the best fit line of the graph shown in Figure 3 is then used to calculate the efficiency (E) using the formula

Table 4. Example dilution series data

Dilution	**Dilution (log_{10})**	**C_t**
1	0	12.82
0.1	-1	16.80
0.01	-2	19.84
0.001	-3	24.18

Figure 2. Example dilution data taken off of the lab manual.

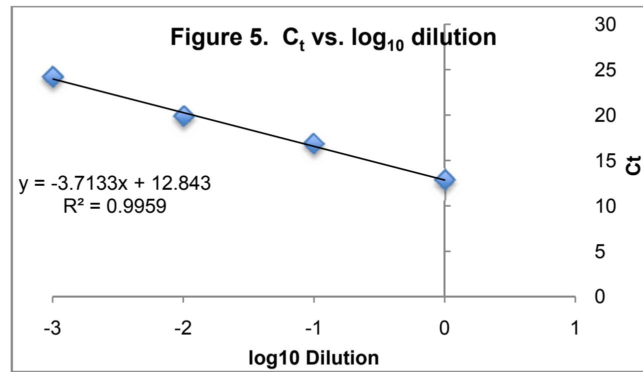

Figure 3. Graph of Cycle Threshold vs. Log10 Dilution, it was taken from the lab manual.

shown below, as equation 1. The reason for this is because this isn't a perfect world and the efficiency isn't exactly 2. Since small changes can drastically affect E, experimentally E must be figured out. One more value must be found in order to calculate the final

$$equation\ 1: E = 10^{(-1/s)}$$

value needed to make the normalized relative abundance bar graphs. When comparing two or more samples, with the same target, the cycle thresholds can be plugged into the equation shown below, labeled as equation 2.

$$equation\ 2: \triangle\ C_t = C_t^{control} - C_t^{experiment}$$

The $\triangle\ C_t$ is then used along with the E to calculate the normalized relative abundance using the final equation represented as equation 3 (Axtell, Burpee and Nelson 2012).

$$equation\ 3: RA_n = (E_{target})^{\triangle C_t\ target}$$

Results:

The excel files that accompany this lab report are where the charts below came from. My specific contribution to the class data was the data shown in the D2, D5 and D6 rows. My samples did work, but my negative did not come out undetermined so I have

some sort of contamination. When comparing the U6 value from the sample or control data to my data I am a bit high, I got a value of 18.25 while the control data was 12.55.

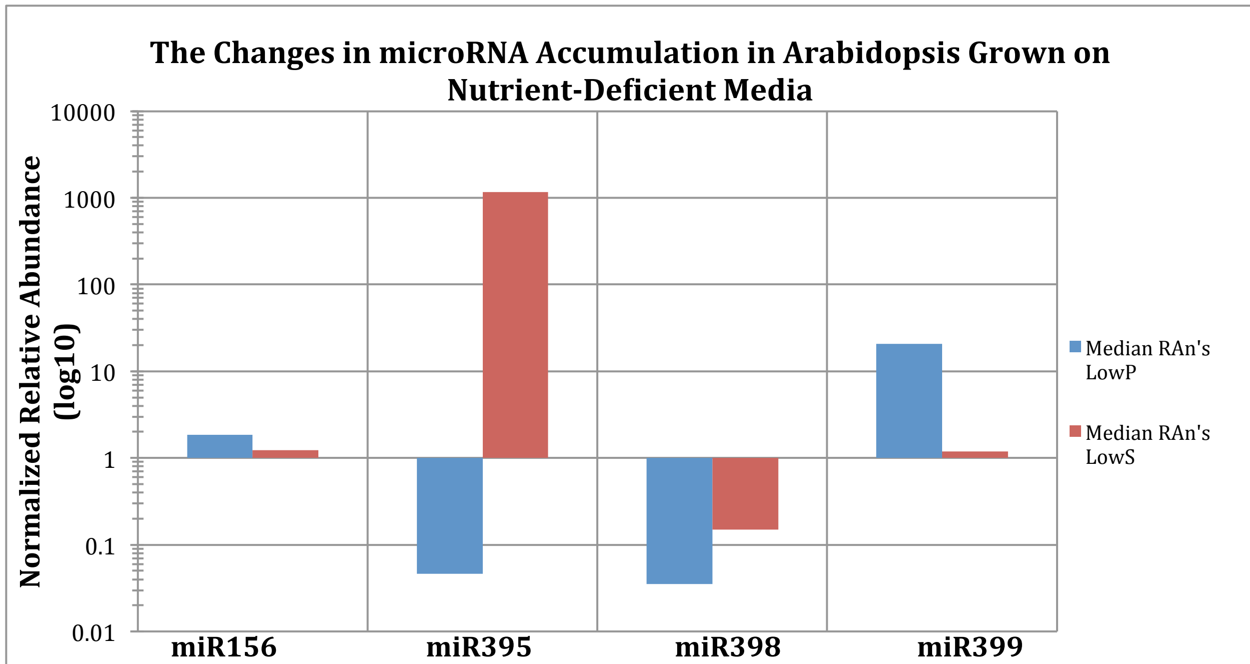

Figure 4. Median values of RAn's from sample data.

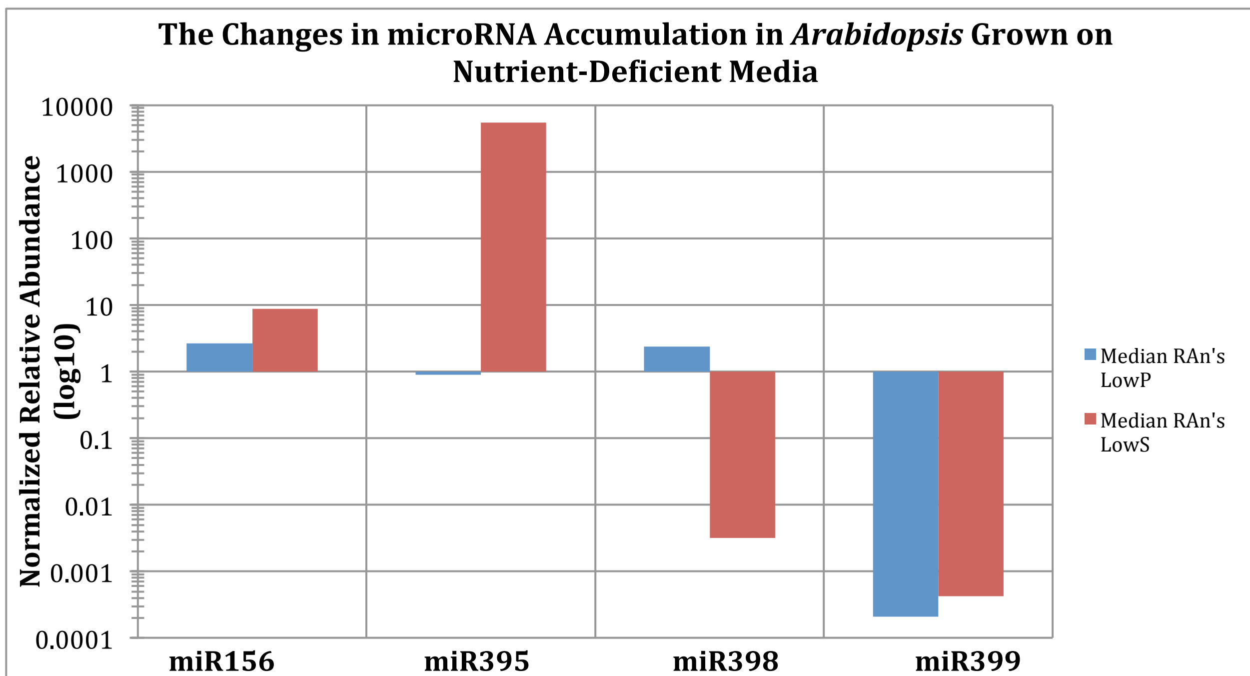

Figure 5. Median values of RAn's from section 029 data.

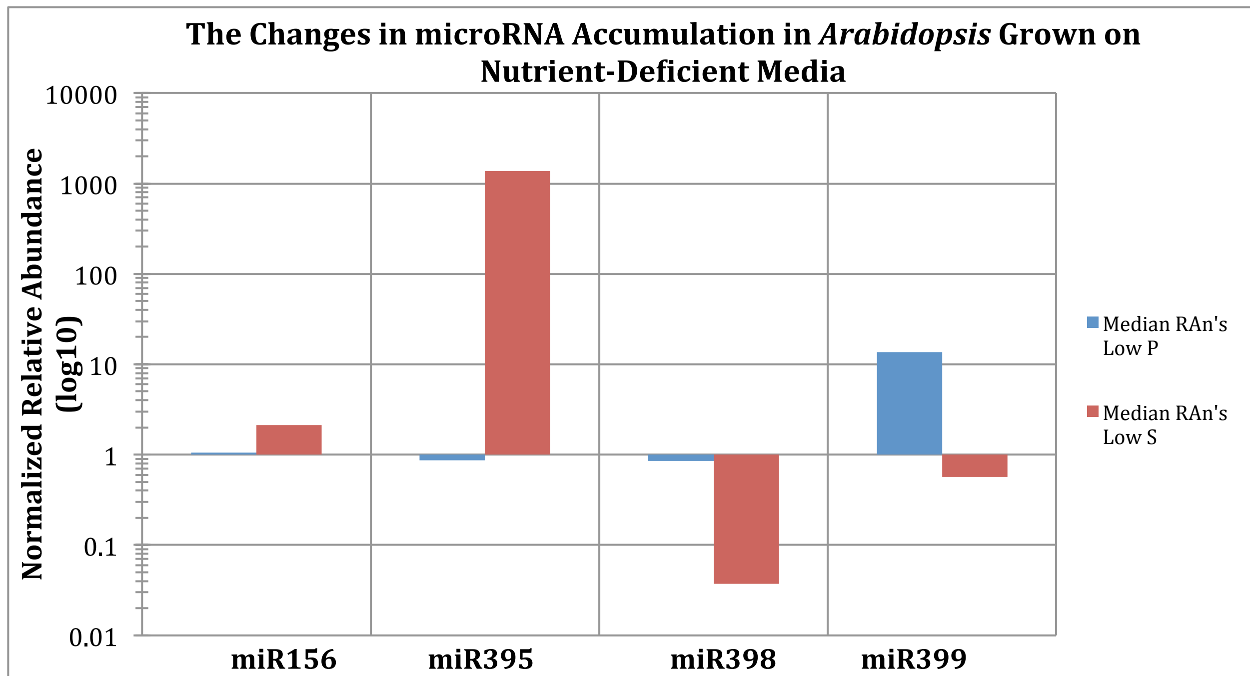

Figure 6. Median values of RAn's from entire Bio 240W class.

Comparisons can be made between all the figures shown above. The sample data in this case is acting as a control data set. First the control data and class data will be compared. The concentration of miR156 in both charts increased in both medias but in the control data the miR156 had a higher increase in the low P while the class data showed a higher increase in the concentration of no sulfur. The miR395 in both graphs showed the highest concentration increase when in the no sulfur media, in contrary the low P media showed a decrease in the concentrations in both graphs, but the control showed a much larger decrease. When comparing miR398 both graphs showed a decrease, but in the control the low P showed a larger decrease while in the class data the no S showed a larger decrease in concentration. In both graphs there is an increase in the concentration of miR399 in low P, but in the control the no S showed a small increase while in class data the no S showed a decrease. Next the control data will be compared to my section data. miR156 showed an increase in both graphs but in the control the low P showed a higher increase however the section data showed the opposite. The highest concentration

increase on either of the graphs was in the miR395 and both were in the no sulfur media, also in both the low P showed a decrease in concentration with the control showing a greater decrease. In both graphs the miR398 showed a decrease in concentration in the no sulfur media, also in the control the low P for miR398 showed a decrease, but the section data showed a slight increase in concentration. Lastly the miR399 were completely different, in both medias of the control there was an increase while the section data showed a drastic decrease in concentration. The final comparison being made will be between the class data and my sections data. The miR156 showed the same pattern in both media, which was an increase. miR395 showed the highest concentration increase in both data tables, the no S in both showed an increase and the low P showed a decrease. In both graphs the no S showed a decrease in miR398, but in the section data the low P showed an increase while the class data showed a decrease. miR399 in both medias of my section data decreased in concentration, however in the class data only no S decreased in concentration, the low P showed an increase.

In general all three graphs showed an increase in miR156 in both media. All three graphs also showed the same trend in the miR395 data, they all showed the highest increase in no S and a decrease in concentration in low P. The trends varied a bit in the miR398 low P medias; the no S in all the graphs showed the same relationship of decreasing but the low P only showed an increase in the section data. miR399 didn't show much of a trend across the different graphs, all were different. Overall there were some trends through the data, which usually suggests some sort of correlation.

Discussion:

The trends seen in figures four, five and six have both similarities and inconsistencies with knowledge that was acquired from articles that have information that relate to the subject of this lab. In all three figures the concentration of miR395 is the highest for no sulfur as the one journal article stated and the highest concentration for the low phosphate media was miR399 for both the section and class data, which is also supported in the article. When the levels of certain nutrients are decreased, in this case sulfur and phosphorus, the miRNA can up or down regulate. In this case the article suggests that the miR395 and miR399 are some of the only microRNAs that up regulate. The majority of the data positively correlates this statement; this means that the initial hypothesis is supported both by the data and the lab article. The reason miR395 shows such a large increase in no sulfur is because miR395 up regulates, this would mean that as sulfur levels decrease, miR395 would increase, all the graphs correlate with this data. From what I could find the no S shouldn't affect the levels of miR395 much, which is supported in figure 5 and 6 but not in figure 4. miR399 according to the lab article also acts as an up regulator and in the case of miR399 it is affected more by low P, which is expected. The graphs from figure 4 and 6 agree with this statement but figure 5 does not, which mean 5 goes against what is expected. A reason that figure 5 might go against the other data is because of possible ingredient errors or contamination, but since the negatives for the miR399 values are undetermined there might not have been any contamination (Chiou, 2007).

There are still two more microRNAs that have to be examined and interpreted, miR398 and miR156. miR398 should show a down regulation in both low P and no S

media, the reason it should work for both is because miR398 down regulates phosphate and nitrogen deficiencies (Zhu, 2010). The reason miR398 also down regulates a sulfur deficiency is because sulfur is directly linked to nitrogen, which is down regulated by miR398 (Hawkesford, Malcolm and Kok). This is supported by the data collected and displayed in figures 4, 5 and 6 but in figure 5 the low P shows an increase. Half of the low P, miR398 was from the data I specifically found. My data actual was contaminated to a degree since the negative, which should show no value, showed a value. This could be the reason why the low P actually showing a up regulation while it should be showing a down regulation.

The last microRNA that was analyzed was miR156 and based on the information found from another lab article from a journal. The lab states that miR156 would up regulate the proteins involved in DNA binding and other larger proteins, since sulfur is involved in proteins and phosphorus is involved with nucleic acids, the decreasing of these would also cause an increase in miR156 (Wang, 2009). Figures 4, 5 and 6 agree with the lab article, all the graphs show an increase. The increases for the most part are low but they are increases never the less, which suggests that the data is supported.

No experiment is without errors, this lab was no exception. The first source of possible error could have been in the micropipetting, when working on such small scales one small pipetting error could cause a dramatic change. Since the pipettes were being shared around the table, there could have been cross contamination. Also with the pipettes, if there were any malfunctions or errors with the pipettes that could have thrown off the accuracy of the volumes being withdrawn. Such changes on such a small scale would cause large changes. I know in my class, there were many people that ran out of the

master mixes, so people had to share, if those that were shared weren't properly made then there could be errors in any samples that used those mixes. All these sources of error, along with others, are the reason that so much of our data was red, which meant it wasn't correct and other data had to be used. The values that we collected and the conclusions that were drawn could be different then the journal articles because of these errors. Most of the conclusions between our data and the journals conclusions were the same, but there was still some variation.

In future labs, there are multiple variations of this lab that could be tested to gain more data. More types of nutrient deficiencies could be tested, for example potassium, calcium or even magnesium to name a few and see how these nutrient deficiencies affect miRNA levels. Another thing to test would be other types of miRNAs and see what they regulate in the *Arabidopsis* when placed in different nutrient deficient medias. This lab is important because by viewing how these different levels of nutrients affect the plant, we can gauge how much of each nutrient to add to the plant to produce the best plant. Farmers and fertilizer companies could use this information to make better fertilizer to grow hardier plants and cause a faster growth rate. This research could increase crop yields and in turn food supplies.

References:

"Basal Plants." *In-class notes.* Biology 240W. Dr. Axtell. 11 Jan. 2012.

Chiou, Tzyy-Jen. "The Role of MicroRNAs in Sensing Nutrient Stress." *Plant, Cell, and Environment* 30 (2007): 323-32

Collins, F. S. (n.d.). *Arabidopsis*. Retrieved 3 18, 2012, from Model Organisms for Biomedical Research: www.nih.gov/science/models/arabidopsis/index.html

Hawkesford, Malcolm J., and Kok L. J. De. *Sulfur in Plants: An ecological Perspective.* Dordrecht, the Netherlands: Springer, 2007.

"microRNA and Plant Nutrition" Axtell, M. Burpee, D. , and Nelson, K. Depertment of Biology, The Pennsylvania State University, University Park, PA. (2012)

Nature 408, 796-815 (14 December 2000) | doi:10.1038/35048692; Received 20 October 2000; Accepted 15 November 2000

"Plant Nutrition." *In-class notes*. Biology 240W. Dr. Axtell. 27 Jan. 2012.

Wang, Jia-Wei. "MiR156-Regulated SPL Transcription Factors Define an Endogenous Flowering Pathway in Arabidopsis Thaliana." *Cell* 138 (2009): 738-49

Zhu, Cheng. "MiR398 and Plant Stress Responses." *Physiologia Plantarum* 143 (2010): 1-9.