Methylome Analysis of Nutrient-Stressed *Brassica rapa*

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Abstract

Epigenetic modifications are at the forefront of agricultural research for crop improvement, especially with the public drive to eliminate chemical fertilizers in crop production. Basic knowledge on plants' responses to lack of nutrients is imperative to drive progress in this direction. In this study, samples of *Brassica rapa* grown in different nutrient stresses are analyzed physiologically by recording height and molecularly by Southern Blot Analysis. Stunted growth along with global methylation level differences indicate that there are differences occurring in gene expression and in DNA methylation simultaneously in plants lacking nutrients.

Introduction

The exposure of an organism to stress throughout its lifetime leads to changes in epigenetic marks, especially DNA methylation (Pareek, Sopory, Bohnert, Gavindjee, 2010). DNA methylation and demethylation, modifications to add or remove a methyl group to the nucleotide base cytosine, both work to regulate an organism's expression of its genes (Pareek, et al., 2010). The rate of change of a plant genome in regards to new gene combinations is relatively slow when compared with the rate of change the environment can achieve. Instead, rapid and dynamic responses to modify the existing genome of a plant, such as DNA methylation, enable the genetic diversity needed to respond and overcome such environmental changes (Peng, Zhang, 2009).

Little is known about plants' specific methylation responses to environmental cues, but the growing interest in agricultural industry to produce crops without chemical fertilizers is fostering the growing interest to understand all aspects of a plant's response to nutrient shortage, including epigenetics. According to North Carolina Department of Agriculture (2010), nitrogen, phosphorus, and potassium are "primary nutrients", three of the most important macronutrients for a plant's growth and development. Nitrogen is the most needed macronutrients by all plants, as it is required for the synthesis of nucleic acids, amino acids, and chlorophyll (Kulcheski, Correa, Gomes, de Lima, Margis, 2015). Phosphorus is one of the less available macronutrients found in soil due to its scarcity particularly in the form orthophosphate, or Pi, which is the only form plants can readily uptake (Kulcheski, et al., 2015). However, phosphorus is still needed in large amounts by plants for proper function of signal cascade pathways, energy metabolism, and enzymatic activities (Kuo, Chiou, 2011). Potassium is a macronutrient also directly involved in crop productivity and crop yield; this mineral is a key player in photosynthesis, enzyme activation, and homeostasis (Pettigrew, 2008). In a natural biological ecosystem, these macronutrients may be recycled by other organisms and therefore maintained in the soil over time (Kulcheski, et al., 2015). However, modern agricultural processes can over exploit soil systems in an effort to produce high yields . Since these three minerals are the first to be depleted from the soil due to plants' rapid absorption to fulfill their nutrient needs, these minerals must then be supplied by the use of chemical fertilizers (Kulcheski, et al., 2015).

Epigenetic research is rapidly picking up interest, and is expanding to include subjects from the bacteria, animal, and plant kingdoms. *Arabidopsis thaliana* is one organism from the plant kingdom whose DNA methylation patterns are beginning to be studied more intently. In

one study, *A. thaliana* samples were exposed to limiting phosphate nutrient solutions, and their methylomes, or their genome's overall methylation modifications, were analyzed (Yong-Villalobos, et al, 2015). The study showed dynamic changes in global methylation in response to the Pi starvation, and that the resultant methylations are associated with the Pi starvation responses exhibited by the plants (Yong-Villalobos, et al, 2015). Overall, this study demonstrated that methylation is required for the developmental and molecular responses of a plant to a phosphorus deficiency in its environment (Yong-Villalobos, et al, 2015).

The closest crop relative to *Arabidopsis thaliana* is *Brassica rapa,* commonly known as the Wisconsin FastPlant or the Rape Mustard, and is part of a genus that is a major contributor to worldwide agricultural and horticultural economies by producing a wide variety of vegetables and plant oils (Tang, Lyons, 2012). *Brassica rapa* has a genome size of 529 MB that is completely sequenced, a rapid life cycle of just 5 weeks, and grows to approximately 16 inches (Tang, Lyons, 2012). Its small genome size and ease of cultivation makes this a suitable model organism for this study at Florida Southern College, while still providing relevance to an actual crop system. If methylation has been shown to play a large role in one macronutrient deficiency response, phosphorus, then perhaps it may also do the same for other key macronutrient deficiencies. This study is aimed to explore the differences in global methylome levels of the plant *Brassica rapa* in response to nutrient deficiencies in each of the three primary nutrients, nitrogen, phosphorus, and potassium. Due to the different cellular functions and pathways each of the three macronutrients are involved in, I predict that global methylation levels will differ between the three deficiencies and that all deficiencies will differ from the control.

Materials and Methods

Mixing of Fertilizers: 1L each of Complete, -K, -N and -P nutrient solutions were mixed using the LaMotte Plant Nutrition Chemical Package according to Dr. Robert W Stegner's instructions in his lab manual *Plant Nutrition Studies,* also provided in the LaMotte Plant Nutrition Chemical Package.

Planting and Growing *Brassica rapa* Samples: Three seeds of Wisconsin Fast Plants® Standard *Brassica rapa* Seed from Carolina Biological Supply Company each were planted in 15 plastic four oz drinking cups filled with standard potting mix, and pushed less than 1 cm below the surface of the soil. Each sample received 65 mL of its specified nutrient solution: 6 samples of complete, 3 samples of -K, 3 samples of -N, and 3 samples of -P. Samples were grown in the Florida Southern College Botany Lab under 24 hour fluorescent light for 9 days, and received water every other day. Growth was recorded on days 4, 6 and 8 by measuring height from soil level to top of the primary stem using a metric ruler. Photographs were taken on day 8 to compare physiology.

Plant DNA Extraction: Carried out using the Bioline Isolate II Plant Genomic DNA Extraction kit, homogenizing cells by using liquid nitrogen and grinding with a mortar and pestle, and using Lysis Buffer PA2. Approximately 0.2g of leaf tissue was used for each sample in DNA Extraction.

Gel Electrophoresis: Carried out on 2 separate 0.8% agarose gels prepared containing 5

microliters each of 10 mg/mL EtBr, 10 microliters of DNA with loading dye was loaded into each well, and gel was run for 60 minutes at 100V in TAE Buffer. Bioline 50bp Hyperladder was used as the molecular marker in lane 1 of both gels.

Southern Blot Analysis: Protocol for the Southern Blot Analysis was followed according to Miltello's method, with modifications (Militello, K.T., 2013). A DotBlot was employed to load DNA samples onto nitrocellulose membrane in concentrations of 14.5ng, 29ng, and 145 ng consistently for all experimental groups. Instead of baked in an oven for 2 hours, the membrane was exposed to UV light in the UV Crosslinker at level 1. Membrane was blocked with 5% w/v nonfat dry milk in TBST for 7 hours at room temperature, then washed 3x for 5 minutes each with 1x TBST, and loaded with a 1:2,500 dilution of primary antibody 5MeC-anti-rabbit IgG from GeneTex in TBST overnight at 0ºC. Membrane was washed 3x for 5 minutes each with 1x TBST, and treated with a 1:10,000 dilution of secondary antibody rabbit-anti-sheep IgG from Thermo Fisher Scientific in TBST for 6 hours at room temperature. Membrane was washed 3x for 5 minutes each with TBST and covered with a 1:5 dilution of alkaline phosphatase chromagen (BCIP/NBT) from Abcam in DI water, rotating for 25 minutes at room temperature. Photos of membrane were taken every 5 minutes while rotating, and again after membrane had dried.

Quantifying Southern Blot Analysis: Photos of dried membrane was uploaded to FIJI APP, converted to 8bit, and inverted. ROI manager was used to circle each spot of DNA sample, and a circle just outside each DNA sample for background measurements. Mean Gray Value

measurement were taken for each circle, and the corresponding background circle values were subtracted from each DNA sample circle values.

Results

Brassica rapa plants grown in each condition had similar observations when photographed, and the growth measurements established a trend of stunted growth among nutrient deficient samples (Figures 1 and 2). The complete samples reached an average height of 5.43 cm, the -K samples reached an average height of 4.43 cm, the -P samples reached an average height of -4.13 cm, and the -N samples reached an average height of 3.07 cm (Figure 3).

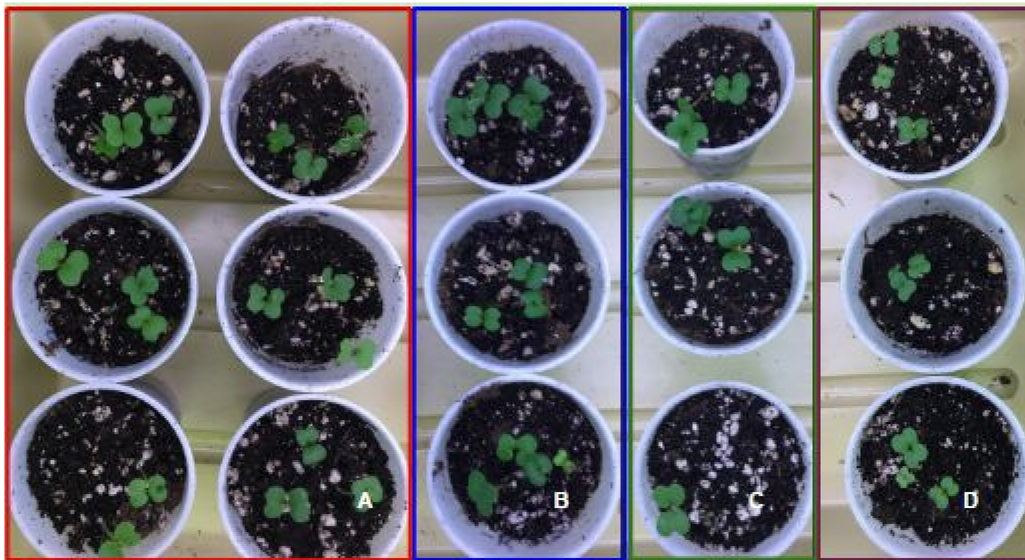

Figure 1: *Brassica rapa* samples grown under control (A, red), -K (B, blue), -P (C, green), and -N (D, purple). Samples in B, C, and D show stunted growth.

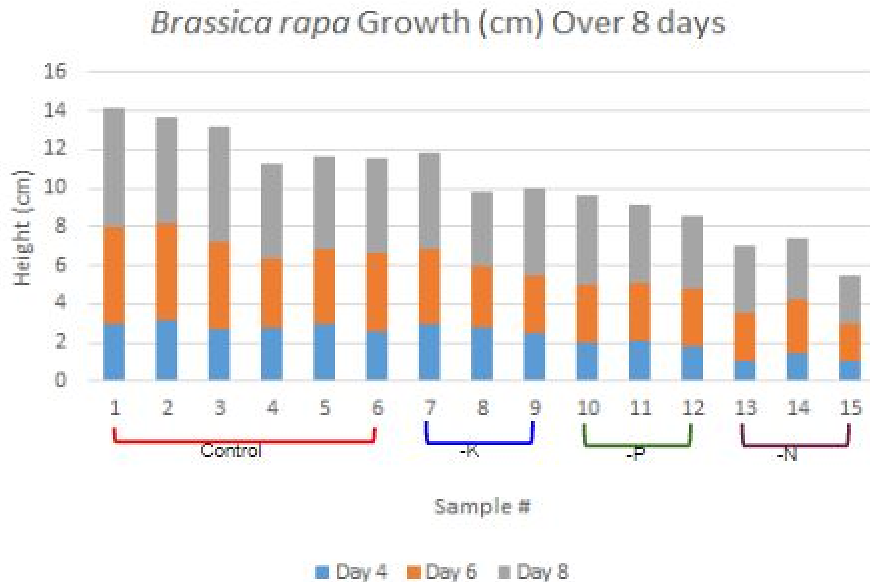

Figure 2: Change in height of Brassica Rapa samples over 8 days. Samples 1-6 were grown with complete nutrient solution, samples 7-9 were grown with -K solution, samples 10-12 were grown with -P solution, and samples 13-15 were grown with -N solution.

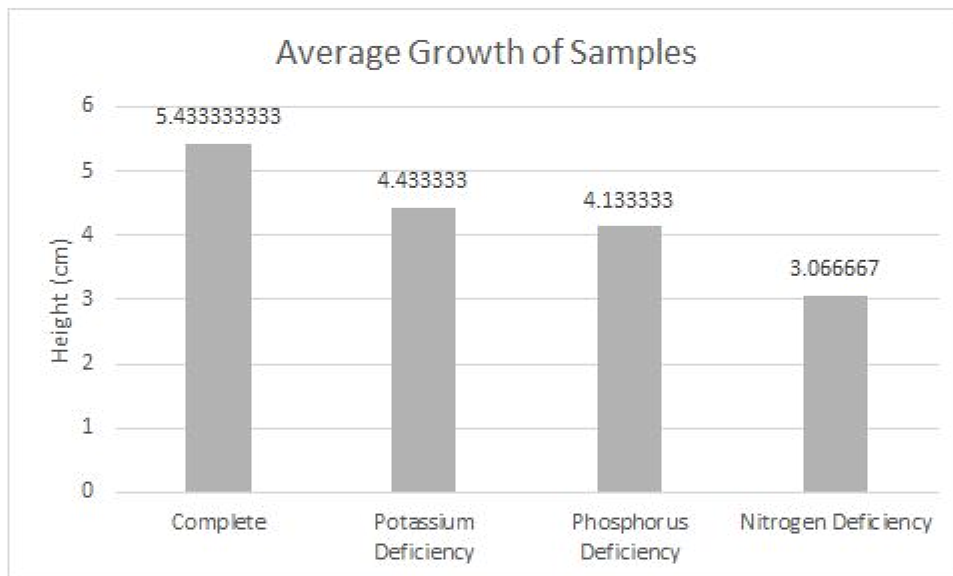

Figure 3: Average Growth of Samples In Each Treatment. Samples grown with complete nutrient solution grew an average height of 5.43 cm, samples grown with -K nutrient solution grew an average height of 4.43 cm, samples grown with -P nutrient solution grew an average height of 4.13 cm, and samples grown with -N nutrient solution grew an average height of 3.07 cm.

Gel electrophoresis of extracted DNA samples showed bright bands in all lanes except for lane 8

(Figure 4).

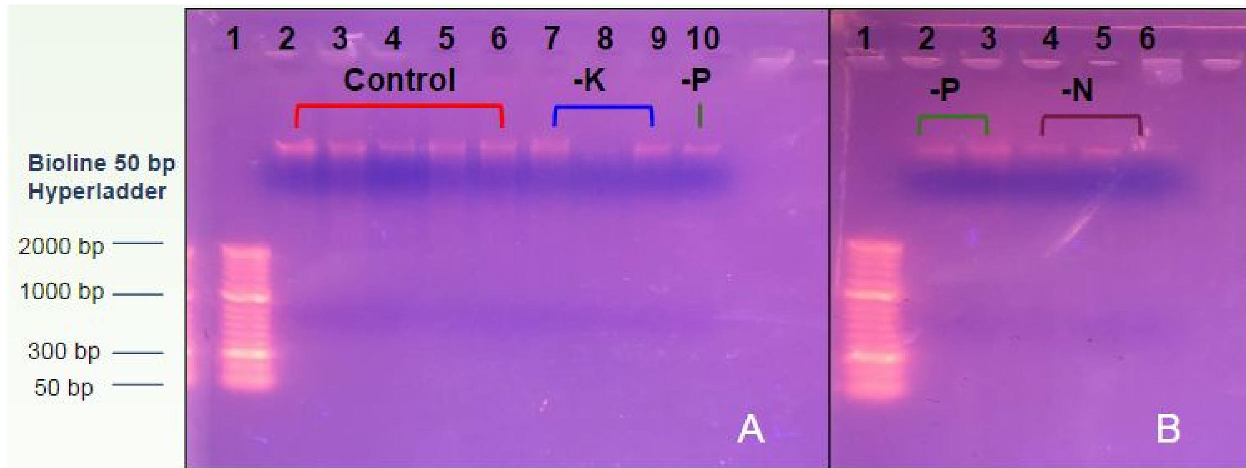

Figure 4: Gel electrophoresis of *Brassica rapa* samples run on 0.8% agarose gel run at 100 V for 60 minutes and Bioline 50 bp HyperLadder loaded into lane 1. Visible bands are shown in all lanes except on gel A, lane 8 shows no visible band. On gel A: lanes 2-6 are extracted from samples grown in complete nutrient solution, lanes 7-9 are extracted from samples grown in -K nutrient solution, and lane 10 is extracted from sample grown in -P nutrient solution. On gel B: lanes 2-3 are extracted from samples grown in -P nutrient solution, and lanes 4-6 are extracted from samples grown in -N nutrient solution.

DNA was obtained from the extraction, and so this was used in the Southern Blot assay. Subtle changes in darkness of spots was observed between the four experimental groups, and increasing darkness trend was observed as concentration of DNA increased for each sample (Figure 5). The FIJI APP Processing provided quantification of darkness observed in Southern Blot (Figure 6). Global methylation measured for samples grown in -K nutrient solution and -N nutrient solution registered lower than the control samples grown in complete nutrient solution, and samples grown in -N nutrient solution registered higher global methylation than control samples grown in complete nutrient solution (Figure 6).

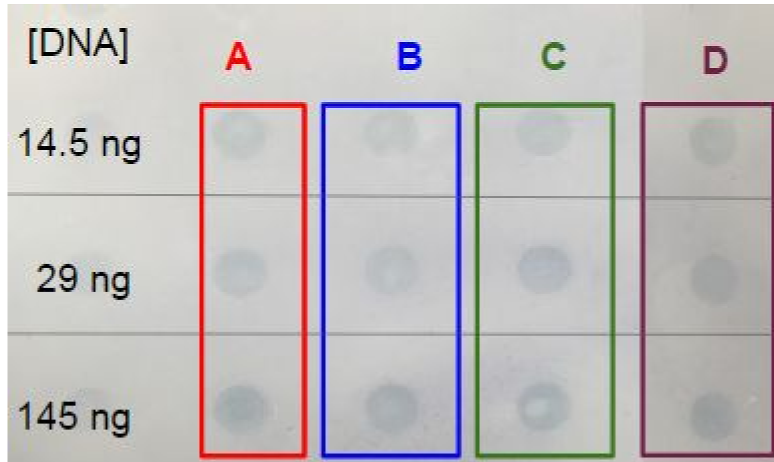

Figure 5: Southern Blot Analysis of Samples for Global Methylation Levels. Concentration of DNA shown to the left of the blots, Control seen in red, -K seen in blue, -P seen in green, and -N seen in purple. Subtle differences in darkness of spots between each experimental group is seen.

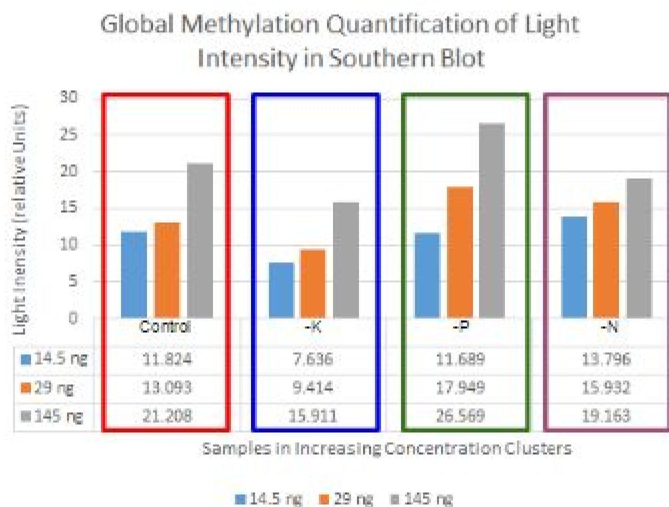

Figure 6: Quantification of Southern Blot using FIJI APP. ROI measurements were taken after photo images of Southern Blot were inverted, background was subtracted, and measurements were organized. Samples grown in control measured to have ROI at 11.824 for 14.5 ng of DNA, 13.093 for samples of 29 ng of DNA, and 21.208 for samples of 145 ng of DNA. Samples grown with -K nutrient solution measured to have ROI at 7.636 for 14.5 ng of DNA, 9.414 for samples of 29 ng of DNA, and 15.911 for samples of 145 ng of DNA. Samples grown with -P nutrient solution measured to have ROI at 11.689 for 14.5 ng of DNA, 17.949 for samples of 29 ng of DNA, and 26.569 for samples of 145 ng of DNA. Samples grown with -N nutrient solution measured to have ROI at 13.796 for 14.5 ng of DNA, 15.932 for samples of 29 ng of DNA, and 19.163 for samples of 145 ng of DNA.

Discussion

This study's main aim to explore the differences in global methylome levels of the plant *Brassica rapa* in response to nutrient deficiencies in each of the three primary nutrients, nitrogen, phosphorus, and potassium. The global methylation levels measured by the Southern Blot and quantified using the FIJI App accomplished this goal, but also warrants further investigation. My predictions at the start of the study were that global methylation levels would differ between the three deficiencies and that all deficiencies will differ from the control, due to the different cellular functions and pathways each of the three macronutrients are involved in. This prediction, too, is supported by the results obtained, but also warrant further investigation as well.

At conception, this study's model organism to be used was *Arabidopsis thaliana*, to be grown in -N and -P nutrient solutions, high light stress, and drought, compared with a control grown in complete nutrient solution. After growing the samples for a semester and attempting to isolate the DNA in one failed attempt, the samples were flash frozen and stored in -80 degrees C freezer for winter break. After several more failed attempts at DNA extraction, despite modification such as more sample, less sample, more incubation time, and more grinding, and no other troubleshooting solutions provided by Bioline technicians, I decided to change the model species from *Arabidopsis thaliana* to *Brassica rapa* and pursue the three macronutrients N, P, and K deficiencies as the experimental groups compared to a control group grown in complete nutrient solution. The high light stress experimental group was not attempted as *Brassica rapa* grows in a 24 hour light period, making this stress obsolete, and drought was not attempted with this species due to its constant photoperiod warranting constant water. From my observations in this study, I would support the idea that *Brassica rapa* is an easy to study model organism for

genetics research.

The samples of *Brassica rapa* grown with each of the four nutrient solutions did not show any physiological differences other than stunted growth (Figures 1 and 2). Stunted growth was seen across all nutrient deficient experimental groups, but was the most severe in the samples grown in -N nutrient solution (Figure 3 and 4). This is no surprise, as nitrogen is the most important macronutrient for all plants, and is needed for many building blocks within plant cells (Kulcheski, et al., 2015). The phosphorus deficiency exhibited the second most severe stunted growth, and this too is expected; the mineral phosphorus is heavily involved in energy metabolism, which is the origin of plant growth (Kuo, Chiou, 2011). Samples grown in Potassium deficient nutrient solutions exhibited the least amount of stunted growth, is surprising considering its direct tie to crop productivity and crop yield in previous studies (Pettigrew, 2008).

The DNA extraction was verified by gel electrophoresis shown in figure 4, with bright genomic bands seen in all lanes except for lane 8 on gel A. This failed DNA extraction attempt can be attributed to improper cell homogenization by grinding with a mortar and pestle in the presence of liquid nitrogen. While grinding 14 samples rapidly in the presence of freezing cold liquid nitrogen, I suspect that I did not pulverize the sample that was loaded into this lane enough in the attempt to finish the grinding of all samples before they began to thaw.

The Southern Blot shows increases in darkness of spot as the concentration of DNA increased for each sample, indicating that there is an increase in global methylation as the amount of DNA increased (figure 5). This helps to verify that DNA was loaded correctly into each circle well, although there are some white spots within the circles in columns A, B, and C.

These variations in color within these circles may be attributed to improper primary or secondary antibody binding due to an air bubble at those spots during their incubation. These spots were taken into account when using the FIJI APP to quantify this data, and they were deleted out of the circles in order to provide more accurate measurements.

The differences between each experimental group at each concentration was very subtle initially, and the use of the FIJI APP allowed for comparisons to be made with more ease. The increase in methylation observed for the samples exposed to -P deficient soil is supported by previous research that observed hypermethylation *Arabidopsis thaliana* samples exposed to low phosphate conditions (Yong-Villalobos, et al, 2015). The similar findings shown in this study within *Brassica rapa* further supports the idea that nutrient stress does have an effect on DNA methylation. The lower levels of global methylation observed in the samples grown in -N and -K compared with the samples grown in complete nutrient solution also supports my predictions made in the beginning of the study. The differences observed in the global methylation levels of all three experimental groups support the idea that the different cellular functions that each macronutrient corresponds to may dictate how much DNA methylation occurs in the absence of each mineral.

Overall, the results of this study support the predictions made initially at the start of the study, but also warrant further investigation. Another qualitative analysis assay of global methylation levels in order to gain more supporting evidence of these trends would greatly benefit this study, such as a restriction enzyme isoschizomer assay outlined in Dr. Militello's paper (Militello, 2013). Additionally, MethyC-Seq library construction and sequencing would be the next step to further this investigation, such as the method outlined in Dr. Yong-Villalobos's

paper (Yong-Villalobos, et al, 2015). This would allow for analysis of where on the genome these methylation are taking place, and which genes they may be affecting, making it possible to investigate further whether the functions each macronutrient is involved in are being altered at the epigenetic level to be expressed differently. The conclusions for this study are preliminary, but show many opportunities to explore in the future. As more knowledge is gained in the field of epigenetic responses of plants to nutrient shortage, high-yield crop production with reduced amounts of fertilizers may one day become a reality.

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