Characterizing Changes in Genome-wide Methylation in Response to Elevated Carbon Dioxide in *Arabidopsis thaliana*: A Methylation Sensitive Amplified Fragment Length Polymorphism (MS-AFLP) Approach

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Abstract

In this study, *Arabidopsis thaliana* (*A. thaliana*) were grown in either ambient carbon dioxide (CO_2) concentrations or elevated CO_2 concentrations where half of the plants from each group were treated with 5-azacytidine during plant development; these treated groups were used as a positive control. At the rosette state, *A. thaliana* leaves were collected for DNA extraction, then were concentrated. The extracted DNA was used for the technique, methylation-sensitive amplified fragment length polymorphism (MS-AFLP) where restriction enzymes, *HpaII* and *MspI* were applied. These restriction enzymes cleave CCGG sites differently and are responsible for identifying differential methylation states throughout the entire *A. thaliana* genome. After the MS-AFLP protocol, the samples were visualized on 4% agarose gels where it was determined that the majority of the CCGG sites were unmethylated, non-polymorphic fragments. Regarding 5-azaC as a positive control, it was successful since more unmethylated sites were visualized in the 5-azaC treated groups, compared to the control groups. As for the differential methylation states, there was evidence that *A. thaliana* growth in different CO_2 concentrations cause differential methylation states since a total of 28 polymorphisms were identified where 10 polymorphisms were identified between control groups grown in either low or high CO_2 while 18 polymorphisms were identified between 5-azaC treated groups grown in either low or high CO_2. Although these polymorphisms were produced, differential methylation seemed to be randomized where patterns among the polymorphisms could not be identified.

Introduction

Evolution, Phenotypic Plasticity, and Evolvability

Evolution by natural selection requires variation, heredity, and fitness differences where genetics is the sole source of variation in populations (Bossdorf, *et al.* 2010; Godfrey-Smith, 2007). Variation in evolution includes different morphologies, physiologies, and behaviors among the individuals within a population (Godfrey-Smith, 2007). Regarding heredity in evolution, traits are capable of being passed from parent to offspring while fitness differences in evolution concern various phenotypes that have different survival and reproduction rates in different environments (Godfrey-Smith, 2007). Evolution by natural selection suggests that plant populations which lack variation, heredity, and fitness differences are unable to evolve, causing the population to remain within a state of stasis. Such a principle also implies that genetic variation is necessary for evolution where genetic variation occurs due to random mutations (Bossdorf, *et al.* 2008; Bossdorf, *et al.* 2010). Low genetic variation makes it more difficult for populations to adapt to different environments as well as affects how populations evolve (Jump and Peñuelas, 2015). Since this is the case, high genetic variation will give populations the ability to adapt or evolve in changing environments. Although evolution by natural selection uses genetic variation as its driving force for phenotypic variation, various studies have shown evidence of phenotypic variation via phenotypic plasticity and epigenetic variation without the assistance of genetic variation (Bossdorf, *et al.* 2010; Nicotra, *et al.* 2010; Pigliucci, 2008).

Phenotypic plasticity is defined as the scope of phenotypes a single genotype can express in various environments (Nicotra, *et al.* 2010; Zhang, *et al.* 2012). Phenotypic plasticity is highly variable in different populations and environments and can affect key functional traits including

height at maturity, flowering time, root-to-shoot ratio as well as several other traits (Nicotra, *et al.* 2010). Also, phenotypic plasticity can affect how plants protect themselves from herbivores or pathogens as well as provide traits that can cause morphological and physiological responses to combat drought (Zhang, *et al.* 2012). Research regarding phenotypic plasticity is important because it will identify for important plastic responses that are affected by different environmental changes including rapid climate change (Nicotra, *et al.* 2010). In the short-term, research will identify plastic responses of current genotypes and help researchers realize which plants will continue to survive under rapid climate change while in the long-term, research will assist in determining which plastic responses may affect plant evolution (Nicotra, *et al.* 2010). Traits which have the potential to increase overall fitness of a genotype due to phenotypic plasticity will likely persist within a population through evolution and will reduce a species' risk of extinction from environmental changes (Nicotra, *et al.* 2010). Evolution and phenotypic plasticity are similar since they are both known to have both genetically controlled and heritable traits, but they differ because evolution causes phenotypic change after several generations while phenotypic plasticity can cause phenotypic changes within one generation (Godfrey-Smith, 2007; Nicotra, *et al.* 2010).

Similar to phenotypic plasticity, evolvability can potentially affect evolution as well (Nicotra, *et al.* 2010; Pigliucci, 2008). Evolvability has several interconnecting definitions (Pigliucci, 2008). Evolvability may be defined as a simpler form of heritability but can also be defined as the idea that genetic architecture and developmental constraints affect the variability of a species so that modifications of long-term adaptation occur and affect the evolution of a species (Pigliucci, 2008). Variability is defined as the degree to which a character is able to vary; this is dependent on the introduction of new genetic variation via mutation or recombination

(Pigliucci, 2008). Elements of evolvability include robustness (where species can accumulate genetic variants, but mutations do not modify phenotype or fitness in a given environment, unless there are environmental changes), modularity (the extent to which phenotypic traits are distinct), and the concept of genotype to phenotype (G → P) mapping function (the production of a phenotype, depending on genetic architecture and how an organism interacted with the external environment during its developmental stages) (Pigliucci, 2008). Such elements suggest that evolvability is different from evolution because evolvability concerns the degree to which a species is able to evolve where G → P mapping function is of great import since it may invoke phenotypic plasticity depending on different environments including rapid climate change (Pigliucci, 2008).

Researchers who believe evolvability to be a simpler form of heritability use heritability as a measure of evolvability, where Flatt said that evolvability can be measured by the capability of a population to respond to selection while Griswold said that the rate of evolution in a character is influenced by heritability (Pigliucci, 2008). Though another researcher, Houle, instead believes the genetic coefficient of variation to be a measure for evolvability instead of heritability (Pigliucci, 2008). Meanwhile, Quayle and Bullock, believe evolvability should be measured through computational science by determining the amount of time it takes for a population to gain a specific phenotypic target, assuming that environmental changes do not shift the phenotypic target (Pigliucci, 2008).

The Effect of Elevated Atmospheric Carbon Dioxide on *Arabidopsis thaliana*

Arabidopsis thaliana (*A. thaliana*) are small, annual, white-flowered plants from the Brassicaceae family (Bossdorf, *et al.* 2010; Zhang, *et al*. 2012). This species begins as seeds,

depending on the genotype, the seeds need to be vernalized (which is defined as the exposure of a seed to cold temperatures while it is germinating to quicken its flowering when it is planted) to allow for flowering (Bossdorf, *et al.* 2010, Diévart and Clark, 2004; Pigliucci, 2002). After vernalization, depending on the plant, the seed is pollinated, which fertilizes the egg and creates an embryo within the silique (Diévart and Clark, 2004). After embryo development, seed germination produces seedlings containing hypocotyl and cotyledons, creating a rosette (Diévart and Clark, 2004). After approximately 45 days, *A. thaliana* develop and create the fully mature plant which contains roots, rosette and cauline leaves, flowers, and silique (fruit) (Bossdorf, *et al.* 2010, Diévart and Clark, 2004). Groups of *A. thaliana* may be separated into three different ecotypes based upon their vernalization requirements. The first group includes late summer annuals which have no response to vernalization and therefore, are expected to reside in habitats that experience harsh winter conditions where *A. thaliana* survive as seeds in the winter and use the cold temperature of winter as an indicator to halt flowering (Pigliucci, 2002). The second group includes winter annuals which begin flowering in 4-6 months without vernalization but may begin flowering about a month earlier if it is exposed to cold temperatures (Pigliucci, 2002). This group spends its winters as rosettes in milder climates and uses the cold from winter as a signal for the rosettes to switch from its vegetative state to its flowering state (Pigliucci, 2002). This type of vernalization is used to give *A. thaliana* a head-start to the warming season since it is a weak competitor (Pigliucci, 2002). The third group includes early summer annuals which delay their time of flowering after exposure to cold temperatures (Pigliucci, 2002). It is predicted that this *A. thaliana* ecotype was a laboratory line, meaning that it developed under fast life cycles and uniform temperatures which may have changed how vernalization affects it; currently it is unknown whether early summer annuals are found in natural habitats (Pigliucci, 2002).

A. thaliana originated from Eurasia, but varieties of its ecotype are currently inhabiting various climatic areas on a global-scale, including the United States, Norway, Libya, England, and other parts of the world which makes it a versatile plant model, especially since there is a variety of both naturally-occurring variants and laboratory-induced mutants (Alonso-Blanco and Koornneef, 2000; Bossdorf, *et al.* 2010; Pigliucci, 1998; Pigliucci, 2002; Stenøien, 2005). *A. thaliana* is the first plant model used in plant genetics, but is commonly used for research in developmental biology, evolutionary biology, and cell biology as well (Pigliucci, 1998; Pigliucci, 2002; Stenøien, 2005; Wienkoop, *et al*. 2010; Zhang, *et al*. 2012). *A. thaliana* is commonly used as a plant model for research due to its ability of selfing which allows natural inbreeding within a population and the production of uniform DNA sequences among the individuals within the population (Clauss and Aarssen, 1994; Pigliucci, 1998; Pigliucci, 2002; Wienkoop, *et al*. 2010). Such an ability lowers the effective recombination rate and allows researchers to differentiate between phenotypic and genetic differences (Clauss and Aarssen, 1994; Pigliucci, 1998; Pigliucci, 2002). Also, *A. thaliana* is a well-known plant model that continues to accumulate information in its growing database since it has been used as a plant model for many years (Pigliucci, 1998; Pigliucci, 2002). Through various studies, information regarding *A. thaliana* growth, development, and physiology has been collected, making it a reliable plant model (Pigliucci, 1998). Also, the entire genome of *A. thaliana* has been sequenced which is useful for genome-wide methylation detection technologies (Wienkoop, *et al*. 2010). Elevated atmospheric CO_2 concentrations can affect several plant taxa including *A. thaliana* (Dietterich, *et al.* 2015; Jagadish, *et al.* 2016; van der Kooi, *et al.* 2016). Specifically, elevated atmospheric CO_2 concentrations can affect DNA methylation of *A. thaliana*, but research concerning this area is limited (Bossdorf, *et al*. 2010). Past studies also show that elevated

atmospheric CO_2 concentrations can reduce the stomatal density and stomatal index of leaves and further decrease stomatal conductance and transpiration rate in *A. thaliana* (Teng, *et al*. 2006; van der Kooi, *et al*. 2016).

Several studies have grown and cared for *A. thaliana* using a variety of methods. As mentioned earlier, some genotypes may need to undergo vernalization to quicken flowering (Pigliucci, 2002). In such a case, one study cold-stratified seeds in the dark at 4℃ before planting (Bossdorf, *et al*. 2010). Afterwards, seeds may be transferred to soil plots or petri dishes, to continue development to the seedling stage (Bossdorf, *et al*. 2010). This particular study used petri dishes as well as filter paper to provide water to the developing seeds before moving the seedlings to individual soil plots for its final stages of development (Bossdorf, *et al*. 2010).

Epigenetic Variation and DNA Methylation

Epigenetics is the study of heritable changes due to molecular mechanisms which cause modifications in gene expression and function as well as affect phenotypes without changing the DNA sequence (Bossdorf, *et al*. 2008; Bossdorf, *et al*. 2010; Nicotra, *et al.* 2010; Zhang, *et al*. 2012). Epigenetic variation occurs through DNA methylation, histone modification, and RNA interference (Bossdorf, *et al*. 2008; Bossdorf, *et al*. 2010; Nicotra, *et al.* 2010; Zhang, *et al*. 2012). DNA methylation is the transfer of a methyl group to the 5'- carbon on the cytosine aromatic ring which causes transcriptional silencing (Bossdorf, *et al*. 2010). This reaction generally involves the transfer of a methyl group to a CpG island which includes CG, CHG, and CHH sites, where H is equivalent to A, C, or T; depending on the plant species, approximately 4-40% of such cytosines can be methylated (Bossdorf, *et al*. 2008; He, *et al.* 2011; Richards, *et al*.

2017). Currently, DNA methylation is the most understood of these mechanisms, but evidence for natural epigenetic variation via DNA methylation in plants, especially in *A. thaliana*, is lacking. Natural epigenetic variation among plants who are subject to climate change implies that environmental changes alone can potentially influence plant evolution without changing the DNA sequence (Bossdorf, *et al*. 2008; Bossdorf, *et al*. 2010; Zhang, *et al*. 2012).

One study that tested DNA methylation in *A. thaliana* demonstrated that the various genotype responses to demethylation were weakly linked to their genetic relatedness implying that epigenetic variation is independent of genetic variation (Bossdorf, *et al*. 2010). This study specifically showed that experimental DNA methylation resulted in reductions in the overall plant performance of *A. thaliana* where there were major delays in flowering time (Bossdorf, *et al*. 2010). Several other studies have shown that natural variation in epigenetic modifications is possible and that some of these variations are heritable, but are not influenced by genetic variation, implying that there is potential for significant microevolution in plants by natural selection through epigenetic modifications (Bossdorf, *et al*. 2008; Bossdorf, *et al*. 2010; Zhang, *et al*. 2012).

Several techniques have been used to screen for DNA methylation. Assays including HPLC- and ELISA-based assays have been used to approximate the amount of methylated cytosines after global methylation of an entire genome (Richards, *et al*. 2017). Bisulfate-sequencing is becoming the preferred technique for screening DNA methylation due to its ability to measure for all cytosines within a target sequence or even in an entire genome (Richards, *et al*. 2017).

Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) can be used to screen for DNA methylation as well. MS-AFLP uses a modified version of the standard AFLP

protocol (a DNA fingerprinting technique) where pairs of methylation-specific restriction enzymes, *HpaII* or *MspI*, are applied to samples to detect for variations in DNA methylation; such a technique is commonly used in ecological epigenetics and may be used in both model and non-model organisms (Bossdorf, *et al*. 2008; Richards, *et al*. 2017; Vos, *et al*. 1995). Different MS-AFLP protocols have been used in past studies. One study used an AFLP protocol where 200 ng of genomic DNA was digested for 3 hours with 10 units of *EcoRI*, 10 units of *MspI* or *HpaII*, and were immediately ligated with 75 mM of *MspI* or *HpaII* adapters and 20 units of T4 DNA ligase for approximately 16-20 hours at 4℃ (Richards, *et al*. 2012). After ligation, pre-selective amplification by PCR is performed where the thermocycler was set for 2 minutes at 75℃ and was set for 20 cycles of 30 seconds for 94℃, 30 seconds for 56℃, and 2 minutes for 75℃, the final extension was set for 30 minutes at 60℃ (Richards, *et al*. 2012). After the pre-selective amplification, two different fluorescently labelled primers (6-carboxy-fluorescein and 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein) were applied to the PCR mixture for selective amplification (Richards, *et al*. 2012). Gel electrophoresis using a 1% agarose gel was used to check if the amplification was successful where both enzymes cleaved at CCGG sequences (Richards, *et al*. 2012). After the DNA was cleaved, four different types of cuts were identified because *MspI* and *HpaII* cleave different sequences depending on the type of cytosine methylation on the sequence (Richards, *et al*. 2012; Wang, *et al*. 2014). The first type occurred when both enzymes cut the restriction site which indicates no methylation as well as shows bands for both enzymes (Richards, *et al*. 2012; Wang, *et al*. 2014). The second type occurred when *HpaII* cuts while *MspI* does not which indicates for hemi-methylation, as well as shows bands under *HpaII*, but not for *MspI* (Richards, *et al*. 2012; Wang, *et al*. 2014). The third type occurred when *MspI* cuts while *HpaII* does not which indicates the site is fully methylated as

well as shows bands under *MspI*, but not underneath *HpaII* (Wang, *et al*. 2014). The fourth type occurred when neither *MspI* nor *HpaII* cuts which indicates for hypermethylation (Wang, *et al*. 2014). Another study used a similar MS-AFLP protocol, but after the pre-selective amplification, this study performed selective radioactive PCR instead of using fluorescently labelled primers during the selective amplification (Cervera, *et al*. 2002).

Relating to research concerning DNA methylation, the chemical demethylation agent, 5-azacytidine (5-azaC), is often used to study the consequences of demethylation. This chemical agent is added during DNA replication to cause partial demethylation of the DNA via inhibition of the enzyme, methyltransferase. More specifically, other studies have shown that 5-azaC binds to DNA methyltransferases via covalent bonds to form nucleoprotein adducts which cause demethylation since less DNA methyltransferases would be available during DNA replication. Also, another study has shown that 5-azaC acts as a cytosine analog that becomes incorporated directly into DNA during DNA replication; partial demethylation occurs when 5-azaC is incorporated because the structure of 5-azaC is nonmethylable. This chemical agent is advantageous since it gives researchers the ability to generate different epigenetic variants of the same genotype (Bossdorf, *et al*. 2008; Bossdorf, *et al*. 2010; Griffin, *et al*. 2016). Also, this method can be used for many genotypes which lets researchers generalize among various genetic backgrounds (Bossdorf, *et al*. 2008; Bossdorf, *et al*. 2010).

Rapid Climate Change and Biodiversity

Rapid climate change is persistently changing the planet through increases in temperatures, increases in catastrophic climatic events, and altered atmospheric composition (IPCC, 2018; Jagadish, *et al.* 2016; Jump and Peñuelas, 2015; Ward and Kelly, 2004). Some

regions are experiencing climatic changes including increases in temperature and increases in catastrophic climatic events more severely compared to other regions of the world (IPCC, 2018; Jagadish, *et al.* 2016; Jump and Peñuelas, 2015; Ward and Kelly, 2004). By 2040, temperatures are expected to project by 1.5°C, raising the risk for drastic heatwaves and heavy rainfall events (IPCC, 2018). Concerning atmospheric composition, there are increases in greenhouse gases, including atmospheric carbon dioxide (CO_2) concentrations, that are occurring at a global-scale and are providing organisms with more access to carbon for photosynthesis (IPCC, 2018; Ward and Kelly, 2004). Natural sources of greenhouse gases include microbe-controlled pathways such as carbon cycles in ruminants and nitrogen cycles in soil where methane (CH_4) and nitrous oxide (NO_2) are produced, respectively (Hedenus, *et al.* 2013). Greenhouse gases are produced by anthropogenic sources as well, where human food consumption of ruminants and dairy contribute to increases in CH_4 and NO_2 (Hedenus, *et al.* 2013). Also, other anthropogenic sources of greenhouse gases including fossil fuel combustion and deforestation contribute to elevating atmospheric CO_2 concentrations (Ward and Kelly, 2004).

It is forecasted that rapid climate change will affect plant migration patterns. Plants are predicted to migrate to higher altitudes and latitudes to remain within their preferred climatic conditions since global temperatures are increasing and higher altitudes and latitudes tend to be cooler compared to the latter (Feeley, *et al.* 2011; Jump and Peñuelas, 2015; Nicotra, *et al.* 2010). For example, a study on Andean trees determined that more Andean genera were migrating upslope than downslope in the Manu National Park in Peru. From those trees, fifty of the most abundant species were found to migrate at a mean rate of +1.1 m elevation $year^{-1}$ (Feeley, *et al.* 2011). From this investigation, researchers determined that tropical species who are incapable of adapting to increasing global temperatures will lose habitat areas due to

competition as well as raise their risk of extinction (Feeley, *et al.* 2011). Similar results are likely to occur to other plant species if rapid climate change persists (Feeley, *et al.* 2011).

Elevated atmospheric CO_2 concentrations were found to lower levels of zinc, iron, and protein concentrations in wheat, barley, and rice (Dietterich, *et al.* 2015). Other studies have obtained decreased nutrition concentrations in plants (e.g. soybeans, potatoes, sorghum) grown in elevated atmospheric CO_2 concentrations as well, though, relatively small sample sizes were used for these studies meaning that their results may not be significant (Dietterich, *et al.* 2015). Elevated atmospheric CO_2 concentrations and drought conditions were manipulated in one study to compare the growth and yield of crops with different carbon fixations (van der Kooi, *et al.* 2016). C_4 crops, plants that increase their CO_2 fixation in their bundle sheath cells (e.g. sorghum, corn), displayed enhanced growth and yield under dry growing conditions (van der Kooi, *et al.* 2016). Meanwhile C_3 crops, plants that do not increase their CO_2 fixation in their bundle sheath cells (e.g. wheat), displayed enhanced growth and yield under both dry and wet growing conditions (van der Kooi, *et al.* 2016). Results from this study imply that plants may adapt to elevated atmospheric CO_2 concentrations, depending on their growing conditions (van der Kooi, *et al.* 2016). Furthermore, this study found that both C_3 and C_4 plants limit their stomatal conductance (the degree to which a stomate opens), which affects photosynthesis, but elevated atmospheric CO_2 concentrations mitigate conflicts relating to decreased stomatal conductance (van der Kooi, *et al.* 2016).

Both elevated CO_2 concentration and increasing temperatures, may affect plant fitness and flowering as well (Jagadish, *et al.* 2016). For example, populations that can adapt to warm climates have a higher probability of surviving than the latter (Jump and Peñuelas, 2015). As a result, decreasing numbers of cold climate populations will cause decreases in fitness among the

surviving populations due to the lack of genetic variation and may result in the possibility of extinction (Jump and Peñuelas, 2015). Along with warm climate populations, plants with short generation time and short lifespan will have a higher likelihood of adapting to rapid climate change compared to plants with long generation time and long lifespan (Jump and Peñuelas, 2015). Such species are less likely to adapt to rapid climate change because compared to species with short generation time and short lifespan, they have delayed reproductive maturity which results in lower amounts of generations within a period of time and lower chances for the emergence of new genotypes in populations (Jump and Peñuelas, 2015). Therefore, annual plants have a higher potential to adapt to rapid climate change due to their short generation time when compared to plants with longer generation times because the short generation times give the plants the ability to evolve quickly (Jump and Peñuelas, 2015).

Due to rapid climate change, there is a higher chance for the loss of plant biodiversity. Habitat fragmentation, due to either natural or anthropogenic causes, tends to lower biodiversity. Habitat fragmentation makes it difficult for plants to maintain gene flow between neighboring populations because habitat fragmentation makes migrating more difficult for plants (Jump and Peñuelas, 2015). As a result, there is less genetic variation as well as less plant biodiversity. Also, it has been shown that climate change is a potent selective pressure for plants because several studies have demonstrated significant adaptive differentiation in plant populations due to climate change (Jump and Peñuelas, 2015). The studies have shown that populations of *Triticum dicoccocoides* (wild emmer wheat) and *Hordeum spontaneum* (wild barley) were grown in various climate conditions (ex. solar radiation, temperature, aridity stress) which resulted in great amounts of adaptive differentiation among the different populations, implying that climatic factors including temperature and water availability, will affect adaptive differentiation among

plants (Jump and Peñuelas, 2015). Climate change is a potent selective pressure for plants, but relatively slow rates of evolution may inhibit some plants from adapting quickly enough to the rapid rate of increasing global temperatures (Jump and Peñuelas, 2015). It is imperative for both biodiversity and genetic diversity to be maintained since both factors give species the ability to adapt to future environmental changes (Jump and Peñuelas, 2015).

This investigation will test how two different *A. thaliana* genotypes respond to either current, ambient CO_2 concentrations or elevated CO_2 concentrations while using 5-azaC as a positive control. Questions that will be addressed include, (1) will elevated atmospheric CO_2 concentrations affect cytosine methylation in DNA and (2) if there are modifications, to what degree will the plant subjects be affected. MS-AFLP markers will be used to determine whether DNA methylation was successful.

Methods and Materials

Growth of *Arabidopsis thaliana*

In this study, the two *A. thaliana* genotypes, Landsberg erecta (Ler) and Columbia (Col) were grown and tested. P1 seeds were grown in Thermo Scientific™ Precision™ Low Temperature BOD Refrigerated Incubators set between 20-22℃ at 50% humidity with an atmospheric CO_2 concentration of 420 parts per million (ppm). The incubators were installed with fluorescent lights where the plants were grown in 16 hours of light and 8 hours of darkness; they were watered as needed. Once the plants began to bear fruit, F1 seeds were collected from the silique and stored. The F1 seeds were selected by maternal line so that suitable sample sizes were grown between each treatment group of each maternal line. A total of ten different maternal lines were selected, five Ler maternal lines and five Col maternal lines. Each maternal line was prepared on separate petri dishes where the seeds were spread evenly onto a filter paper saturated with deionized water. After placing the seeds on the saturated filter paper, the petri dish was sealed with parafilm and labelled accordingly based on genotype and maternal line; the seeds were vernalized for one week at 4℃. After vernalization, the seeds were separated to create four different treatment groups which include low CO_2 and treated with deionized water, high CO_2 and treated with deionized water, low CO_2 and treated with 5-azaC (75 µM), and high CO_2 and treated with 5-azaC (75 µM). For each petri dish, the filter paper was separated into quarters using a sterilized blade where each quarter contained approximately the same amount of seeds. Afterwards, each quarter was placed into new separate petri dishes which contained a filter paper that was either saturated with 1.5 ml of deionized water or 1.5 ml of 5-azaC (75 µM). All the petri dishes were raised in the incubators where two of the treatment groups were grown in

ambient atmospheric CO_2 concentrations (420 ppm) while the other two were grown in elevated atmospheric CO_2 concentrations (840 ppm) (Figure 1). The same temperature, humidity, and light exposure incubator settings utilized for the P1 seeds were used for the F1 seeds as well. Once the seeds germinated and reached the seedling stage, for one petri dish, the seedlings were transferred to soil pots to continue developing where a total of 18 seedlings were transferred to six different soil pots so that three seedlings were transferred to one soil pot (Figure 2); the seedlings from the other three treatments were transferred to soil pots in a similar fashion as well.

DNA Extraction

At the rosette stage, Col plant samples of approximately 100 mg for each plant were collected for DNA extraction using the protocol for the GenElute™ Plant Genomic DNA Miniprep Kit, but was slightly modified where a bead mill and glass beads were used in place of liquid nitrogen, a mortar, and a pestle to disrupt the cells. Unfortunately, there was not enough Ler plant material for DNA extraction so the majority of the extracted DNA was solely extracted from Col plants. Before DNA extraction, the provided wash solution was diluted with 330 mL of 95-100% ethanol, then was tightly capped and set aside. For DNA extraction, the cells of the plant sample were disrupted and lysed. The plant sample was placed in a microcentrifuge tube containing 3 glass beads along with 350 µl of Lysis Solution Part A, then was grinded in a Fisherbrand™ Bead Mill 4 Homogenizer at 3 m/s for 120 seconds. Then, 50 µl of Lysis Solution Part B was added to the tube and was homogenized again. After homogenizing the sample, a Bio Rad Digital Dry Bath was used to incubate the sample at 65°C for 10 minutes with occasional inversions for cell lysis, then was precipitated where 130 µl of Precipitation Solution was added

to the sample; it was inverted to combine the sample and solution and was placed on ice for 5 minutes. Using a Bio Rad Model 16K Microcentrifuge, the sample was centrifuged at approximately 13-14 RPM for 5 minutes to pellet any cellular debris, proteins, and polysaccharides. After cell lysis, the debris were filtered where the supernatant was pipetted onto a GenElute™ filtration column, then centrifuged at maximum speed for 1 minute. The sample was prepared for binding by adding and inverting 700 µl of Binding Solution with the flow-through containing the supernatant from the previous step. A binding column was prepared by placing a GenElute™ Miniprep Binding column into a centrifuge tube, then 500 µl of Colum Preparation Solution was added to each miniprep column. The tube was centrifuged at approximately 13-14 RPM for about 30 seconds, then the flow-through was discarded. A measurement of 700 µl of the sample was pipetted into the prepared binding column and was centrifuged at maximum speed for 1 minute, then the flow-through was discarded. Using the same collection tube, the remaining amount from the sample was added to the column and centrifuged again. The flow-through was discarded after centrifugation. To wash the column, the binding column was transferred to a new 2 mL tube and 500 µl of diluted Wash Solution was added to the column. The column was centrifuged at maximum speed for 1 minute and the flow-through was discarded. Another 500 µl of diluted Wash solution was added to the column and centrifuged at maximum speed for 3 minutes. The binding column was placed into a new 2 mL collection tube to elute the DNA. An amount of 100 µl of pre-warmed (65°C) Elution Solution was added to the column and centrifuged at maximum speed for 1 minute. Lastly, the elution was repeated, and samples were stored at -20°C. After DNA extraction, the extracted DNA was placed into a vacufuge to increase the DNA concentration of each sample, then the samples were prepared for the MS-AFLP protocol.

Methylation-Sensitive Amplified Fragment Length Polymorphism (MS-AFLP)

A total of 14 different plants were used for the MS-AFLP protocol where only the extracted DNA from Col plant samples were used for the MS-AFLP protocol since there was low Ler plant material and therefore, low amounts of extracted DNA. There were three plants grown as controls in low CO_2 (LC), three plants grown as controls in high CO_2 (HC), four plants grown as 5-azaC treated in low CO_2 (LA), and four plants grown as 5-azaC treated in high CO_2 (HA). First, the extracted DNA samples were prepared for the single digest. Each sample was divided into two different portions each, which both contained approximately 1000 µg of DNA. Then, each portion was combined with 5 µl of 10X CutSmart buffer, 2 µl of either *HpaII* (20KU/mL) or *MspI* (20KU/mL), and nuclease free water (NFW) where the amount of NFW was adjusted so that each single digest portion consisted of a total volume of 50 µl. Afterwards, the portions were flicked and spun down with a Bio Rad Mini Micro Centrifuge to thoroughly combine the contents, then were digested at 37℃ for 1 hour and inactivated at 65℃ for 20 minutes using a Bio Rad T100™ Thermal Cycler. Second, the samples were prepared for a second digest where 2 µl of *EcoRI* – HF was applied to each sample, then was digested and inactivated using the same protocol as the previous digest and inactivation step using the thermocycler. Third, each double-digested sample was prepared for adapter ligation where 14 µl of digested product, 12 µl of NFW, 4 µl of 10X T4 ligase buffer, 4 µl of *EcoRI* adapter, 4 µl of *Hpa*/*Msp* adapter, and 2 µl of T4 ligase was combined. The samples were flicked and spun down to thoroughly combine the content, then incubated at room temperature for at least 15 minutes and deactivated 65℃ for 20 minutes using the thermocycler. Fourth, each digested/ligated sample was prepared for preamplification PCR where all the samples were diluted 5-fold. Next the preamplification mixtures were prepared for each sample and contained 16 µl of 5-fold

diluted digested/ligated product, 5 µl of NFW, 2 µl of *EcoRI* primer + A (10uM), 2 µl of *Hpa*/*Msp* primer + A (10uM), and 25 µl of *Taq* 5X Master Mix Hot Start. The samples were flicked and spun down to thoroughly combine the content, then amplified using the following PCR protocol, 94℃ for 3 minutes, 94℃ for 30 seconds, 58℃ for 1 minute, 72℃ for 1 minute, repeat step 2 and steps after it, 35 times, 72℃ for 5 minutes, and 4℃ for infinite hold. Fifth, the resulting preamplification PCR products were prepared for selective PCR. Each selective PCR mixture contained 3 µl of preamplification PCR product, 18 µl of NFW, 25 µl of *Taq* 5X Master Mix Hot Start, 2 µl of *EcoRI* primer + NN (10uM), and 2 µl of *Hpa*/*Msp* primer + NNN (10uM) where different primer pair combinations were applied to each selective PCR mixture (Table 1). The selective PCR mixtures were flicked and spun down to thoroughly combine the contents, then were amplified using the previous PCR protocol for the preamplification PCR step. After the PCR reactions, the selective PCR products were prepared for visualization using a 4% agarose gel for gel electrophoresis to detect for DNA methylation.

Agarose Gel Electrophoresis

For gel electrophoresis, small-sized agarose gels were prepared using 2 g of Fisher BioReagents™ agarose and 50 mL of 10X Carolina Tris Borate EDTA Buffer which consisted of a total of 16 wells where there were 8 wells for each row. Each loading sample was prepared with 10 µl of selective PCR product and 1.5 µl of Midori Green Direct, then was flicked and spun down to combine the contents. A total of approximately 10 - 10.5 µl of one loading sample was applied to each well, then the gel ran for approximately 1 hour and 20 minutes at 120V. The gels were visualized for scoring using a blue UV light.

Gel Scoring

The agarose gels were scored to determine the methylation state of each site by following the cut identification types by Wang, *et al*. (2014), but the fourth type of cut was excluded from quantitative data to simplify the scoring procedure so that only three different methylation states were calculated. Although, the fourth type was included in the qualitative data since it appeared in some of the polymorphisms. Therefore, corresponding fragments that appeared after being digested by both *HpaII*/*EcoRI* and *MspI*/*EcoRI* indicated for unmethylated sites, fragments that appeared after being digested with *HpaII*/*EcoRI* indicate for hemi-methylated sites, and fragments that appeared after being digested with *MspI*/*EcoRI* indicate for methylated sites. Also, if fragments appeared in one treatment, but were absence in the corresponding area of another treatment, then it implies that there was hypermethylation.

Results

A total of 123 bands were scored among the four different *A. thaliana* treatments. Figure 3 shows the different proportions of methylation states among the different treatments while Table 2 shows the different proportions of methylation states among the different treatments using percentage values. Tables 3, 4, and 5 show the different methylation states among the different treatment groups where the figures were grouped based upon the primer pair combination that were applied to them during the selective PCR. Table 6 shows treatment groups containing differentially methylated polymorphic fragments. Figure 4 through Figure 14 are agarose gels that were being visualized using a blue light; the colored arrows point at fragments and their corresponding areas to identify for differential methylation sites where different colors are used to easily compare the same fragments and their corresponding areas between the treatment groups.

Tables

Primer Combinations		
Primer pairs (PP)	***EcoRI* primer**	**HpaII/MspI primer**
1	*EcoRI* + AC	*HpaII/MspI* + AAT
2	*EcoRI* + AT	*HpaII/MspI* + AAT
3	*EcoRI* + AA	*HpaII/MspI* + ACT

(Table 1: Primer combinations using EcoRI and HpaII/MspI primers with selective nucleotides that were applied during selective PCR)

	Unmethylated	**Hemi-methylated**	**Methylated**	**Ambiguous**
LC	69.74%	11.67%	4.71%	14.80%
HC	61.16%	5.70%	9.71%	23.43%
LA	79.93%	7.28%	7.12%	6.47%
HA	75.37%	4.32%	10.79%	9.52%

(Table 2: Table showing differential methylation states among the different *A. thaliana* treatments where "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)

	Figure 4	Figure 7	Figure 10	Figure 13
LC	UM	HPM	HPM	
HC	UM	HPM	M	
LA	HM	HM	UM	HPM
HA	M	M	HM	M
LC		HM	HPM	
HC		HPM	HPM	
LA		HPM	M	
HA		HPM	M	
LC		HM	M	
HC		HPM	M	
LA		HPM	M	
HA		HPM	UM	
LC		HPM	M	
HC		M	M	
LA		HPM	HM	
HA		HPM	HPM	
LC		HPM		
HC		HM		
LA		HPM		
HA		HPM		
LC		M		
HC		M		
LA		HPM		
HA		UM		
LC		M		
HC		HPM		
LA		M		
HA		M		

(Table 3: A table showing the different methylation states that were identified after scoring agarose gels that were digested by PP_1."LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2. "UM" indicates for unmethylation, "HM" indicates for hemi-methylation, "M" indicates for methylation, and "HPM" indicates for hypermethylation. Each section was color-coded to match the corresponding arrows on each figure)

	Figure 5	Figure 8	Figure 11	Figure 14
LC	HPM	HPM	HM	
HC	HPM	M	HPM	
LA	M	M	HPM	UM
HA	M	HPM	HPM	M
LC	HM	UM	HPM	
HC	HM	A	HPM	
LA	M	UM	M	
HA	M	A	M	
LC	HM			
HC	HM			
LA	HPM			
HA	HPM			

(Table 4: A table showing the different methylation states that were identified after scoring agarose gels that were digested by PP_2."LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2. "UM" indicates for unmethylation, "HM" indicates for hemi-methylation, "M" indicates for methylation, "HPM" indicates for hypermethylation, and "A" indicates for ambiguous. Each section was color-coded to match the corresponding arrows on each figure)

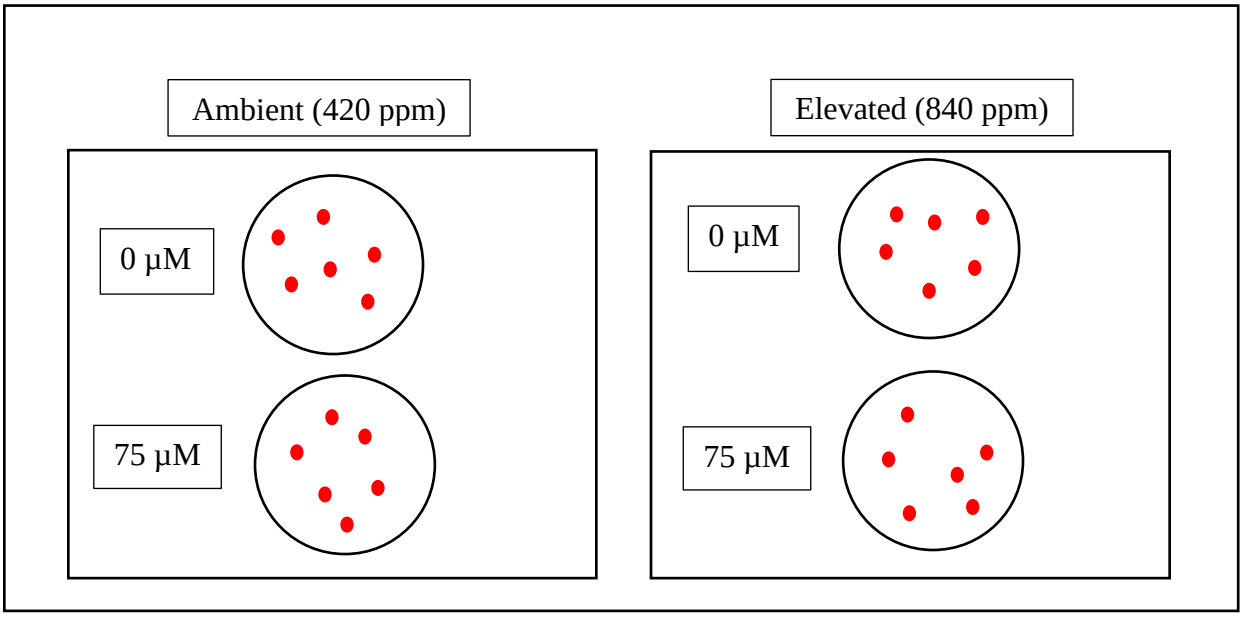
	Figure 6	Figure 9	Figure 12	Figure 13
LC	HM	HM	HM	
HC	UM	HM	HM	
LA	HM	HPM	HM	HM
HA	UM	HPM	HM	HM
LC	HM	M	HM	
HC	HM	M	HM	
LA	HM	HM	HM	HPM
HA	UM	UM	HM	UM
LC	UM	HPM	HM	
HC	HM	HPM	HM	
LA	HM	HPM	HM	HPM
HA	UM	M	HM	UM
LC		HM		
HC		HPM		
LA		HPM		
HA		HM		
LC		HPM		
HC		HPM		
LA		M		
HA		M		

(Table 5: A table showing the different methylation states that were identified after scoring agarose gels that were digested by PP_3."LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2. "UM" indicates for unmethylation, "HM" indicates for hemi-methylation, "M" indicates for methylation, and "HPM" indicates for hypermethylation. Each section was color-coded to match the corresponding arrows on each figure)

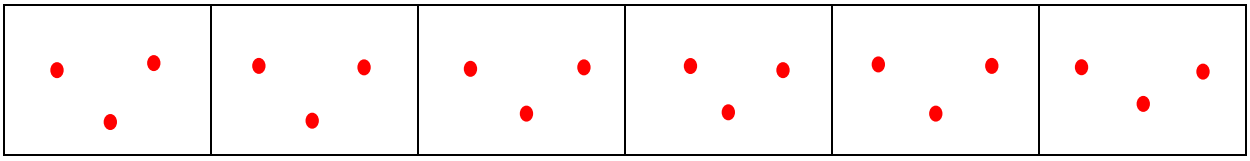
Differential methylated polymorphisms	Control	5-azaC treated
M → HM	0	0
HM → M	0	2
UM → HM	1	1
HM → UM	1	4
M → UM	0	1
UM → M	0	2
M → HPM	0	1
HPM → M	3	2
HM → HPM	3	2
HPM → HM	2	0
UM → HPM	0	0
HPM → UM	0	3

(Table 6: Table that summarizes the different polymorphisms identified between low CO_2 and high CO_2 where the polymorphisms are described as changes in methylation states from low CO_2 to high CO_2; "UM" indicates for unmethylation, "HM" indicates for hemi-methylation, "M" indicates for methylation, and "HPM" indicates for hypermethylation)

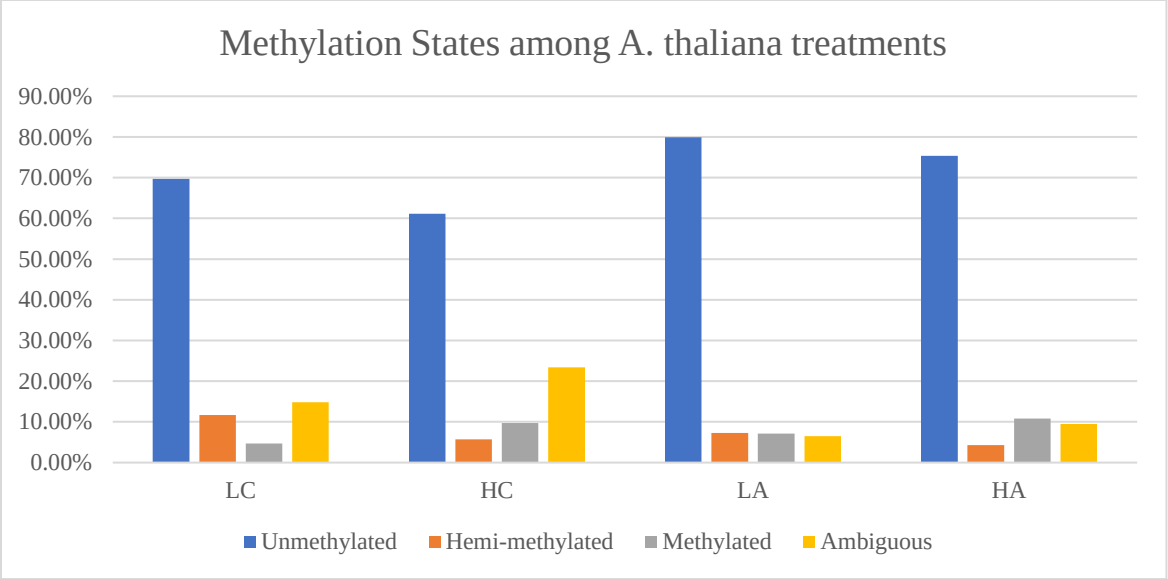
Figures



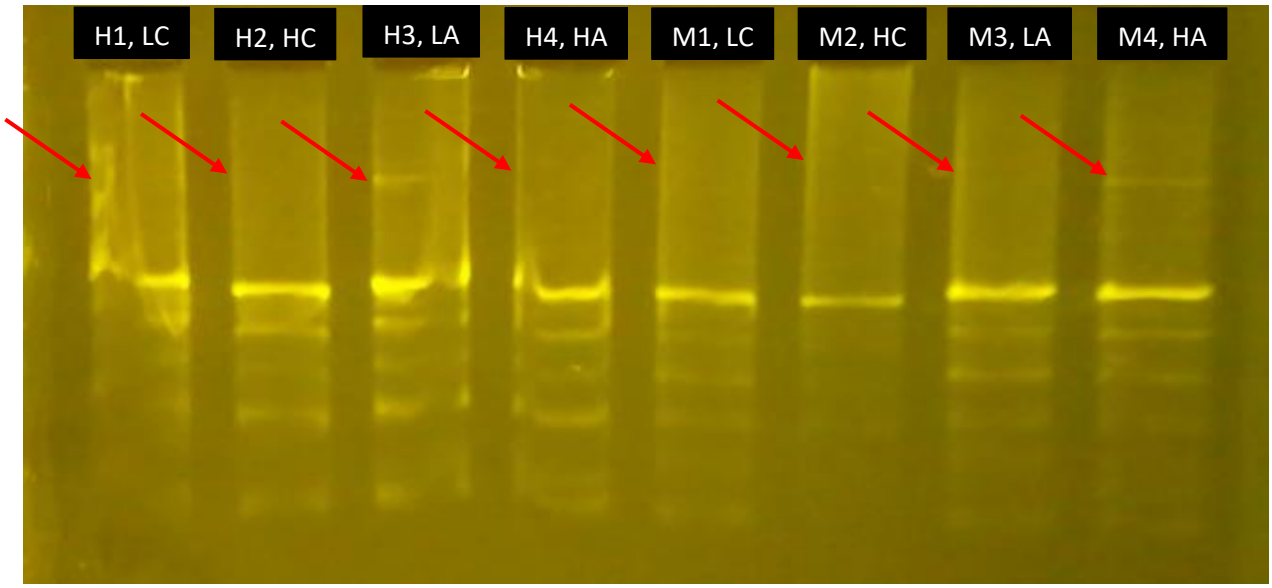
(Figure 1: Diagram of the lab setup of one genotype for this investigation where the circles are the petri dishes and the red marks are seeds)



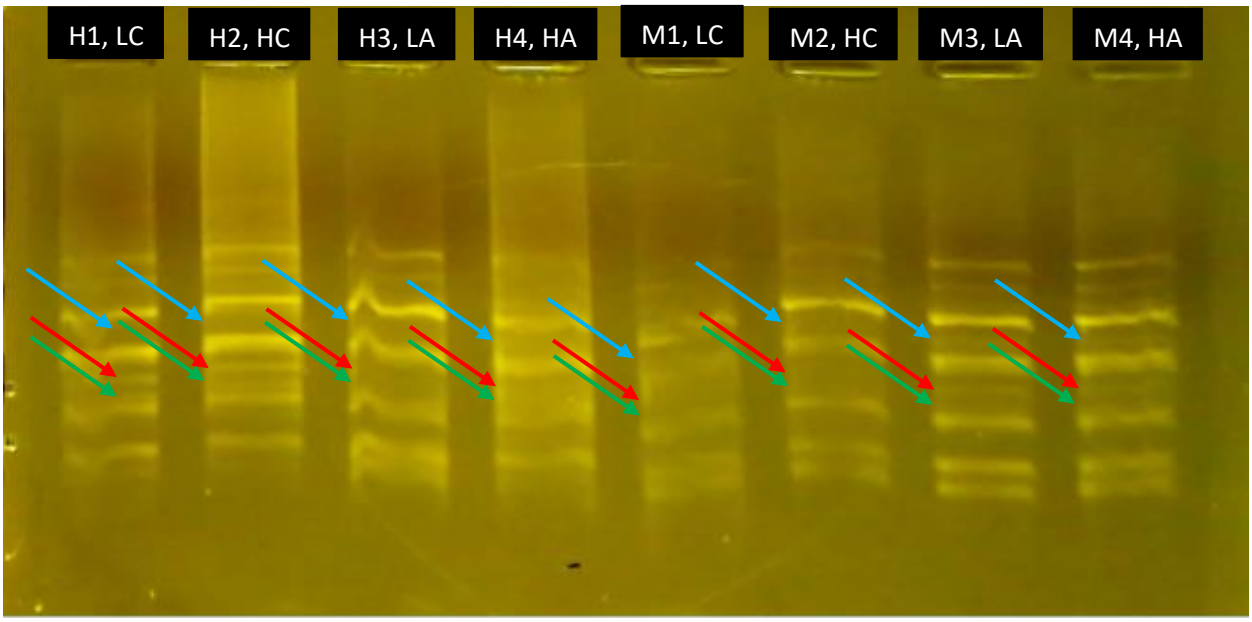
(Figure 2: Diagram of the soil pot set up for one treatment group where the red marks represent seedlings and each row contained a different treatment group so that there was a total of 16 different treatment groups)



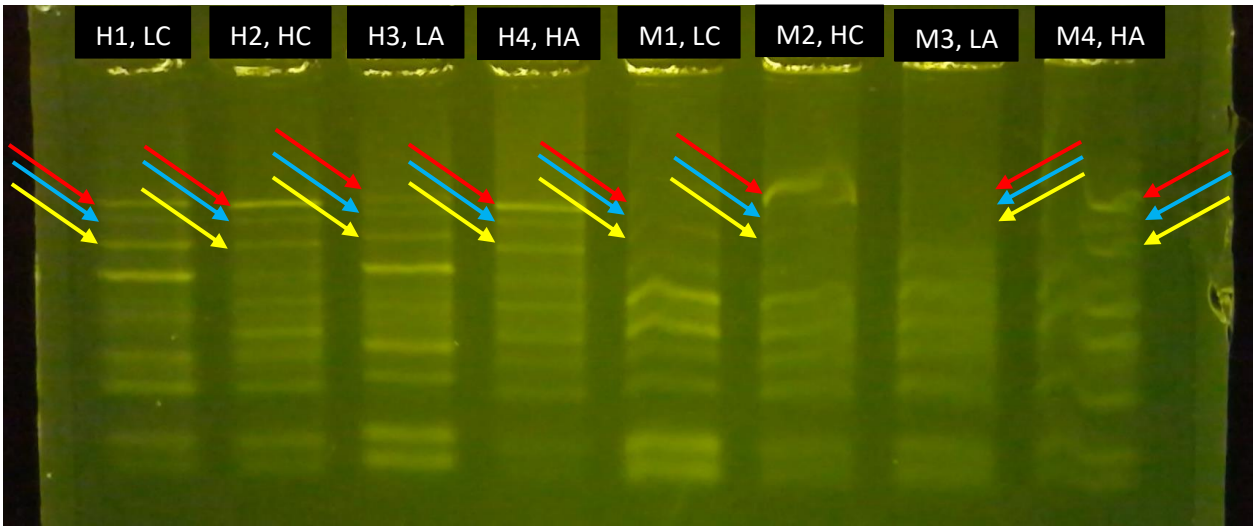
(Figure 3: Bar graph showing differential methylation states among the different *A. thaliana* treatments where "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



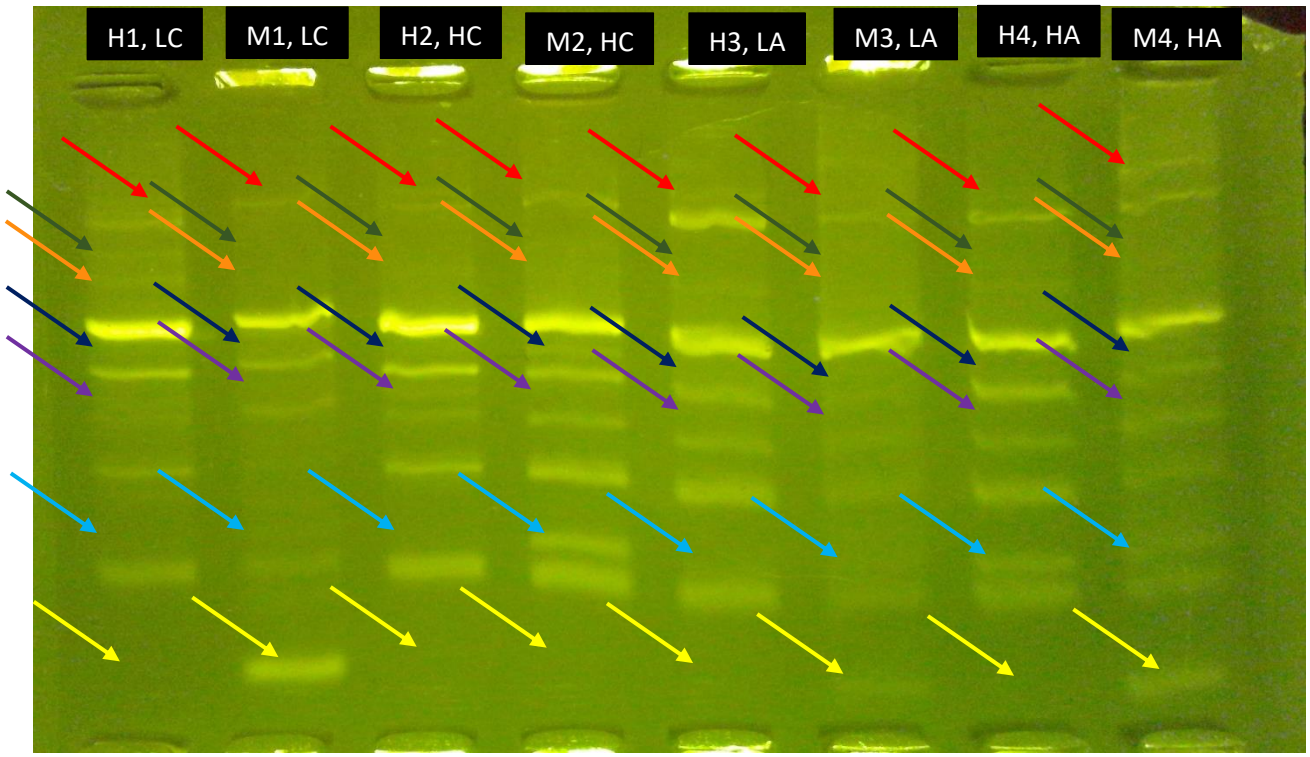
(Figure 4: Agarose gel of fragments amplified by PP_1. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



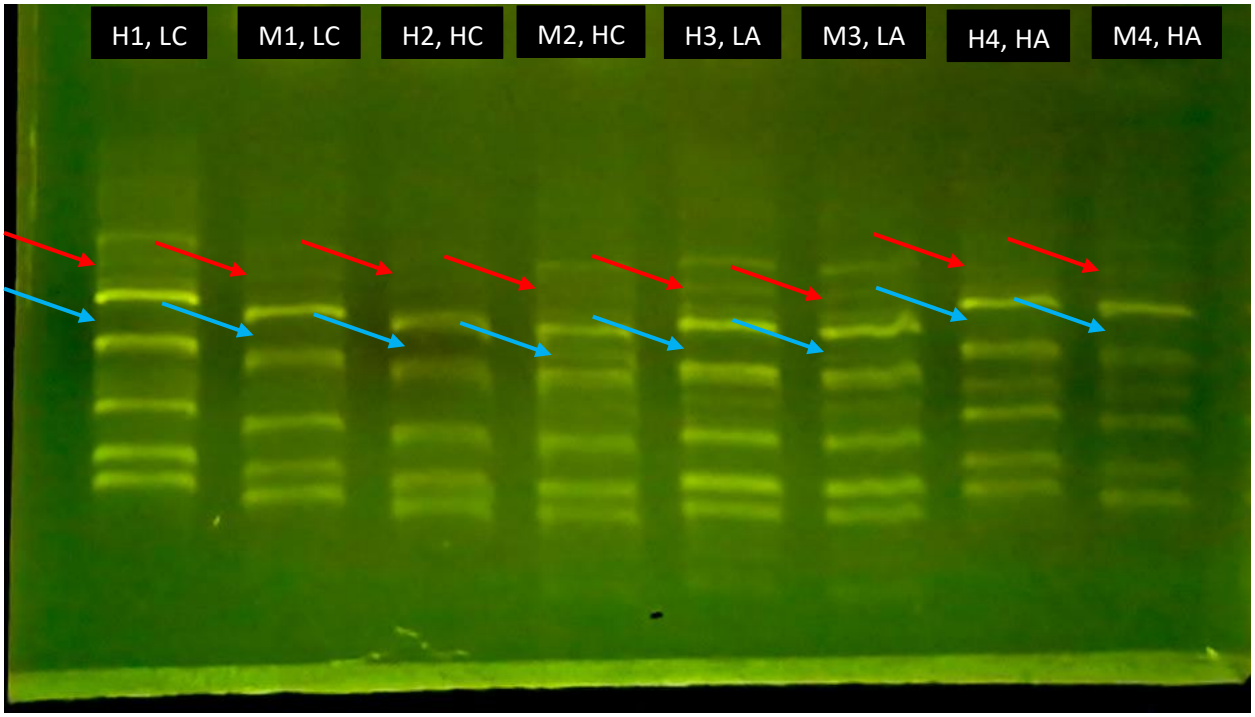
(Figure 5: Agarose gel of fragments amplified by PP_2. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



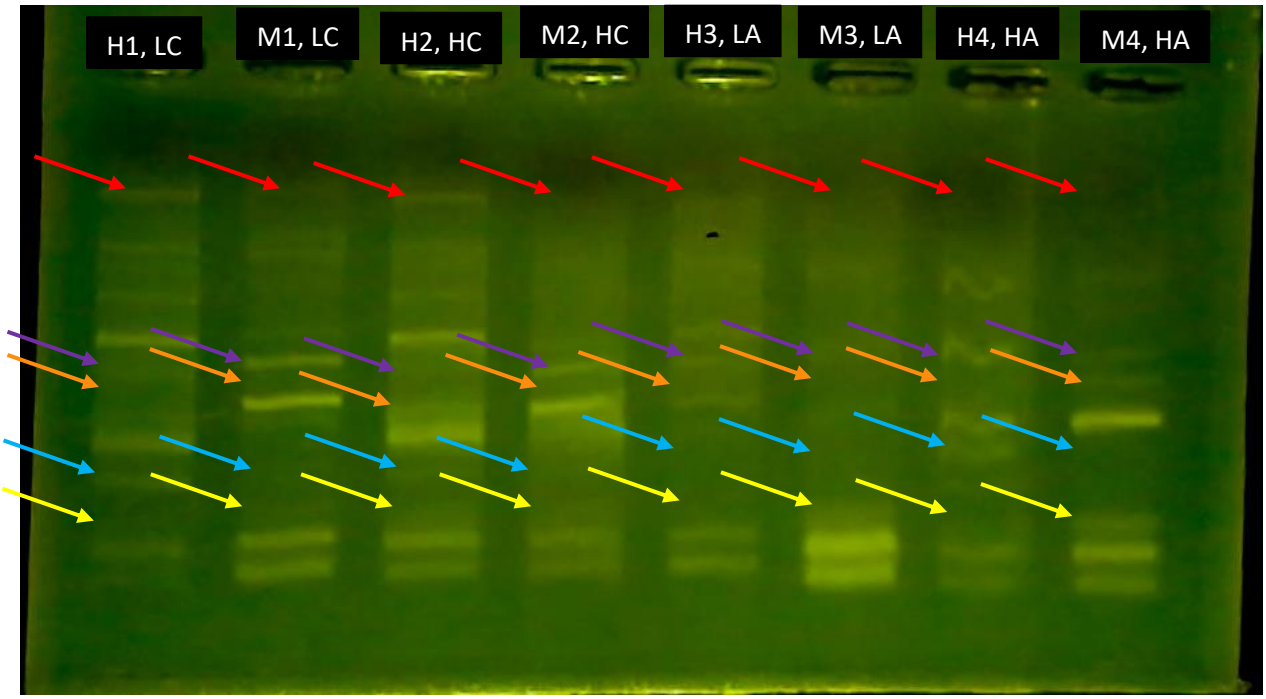
(Figure 6: Agarose gel of fragments amplified by PP_3. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



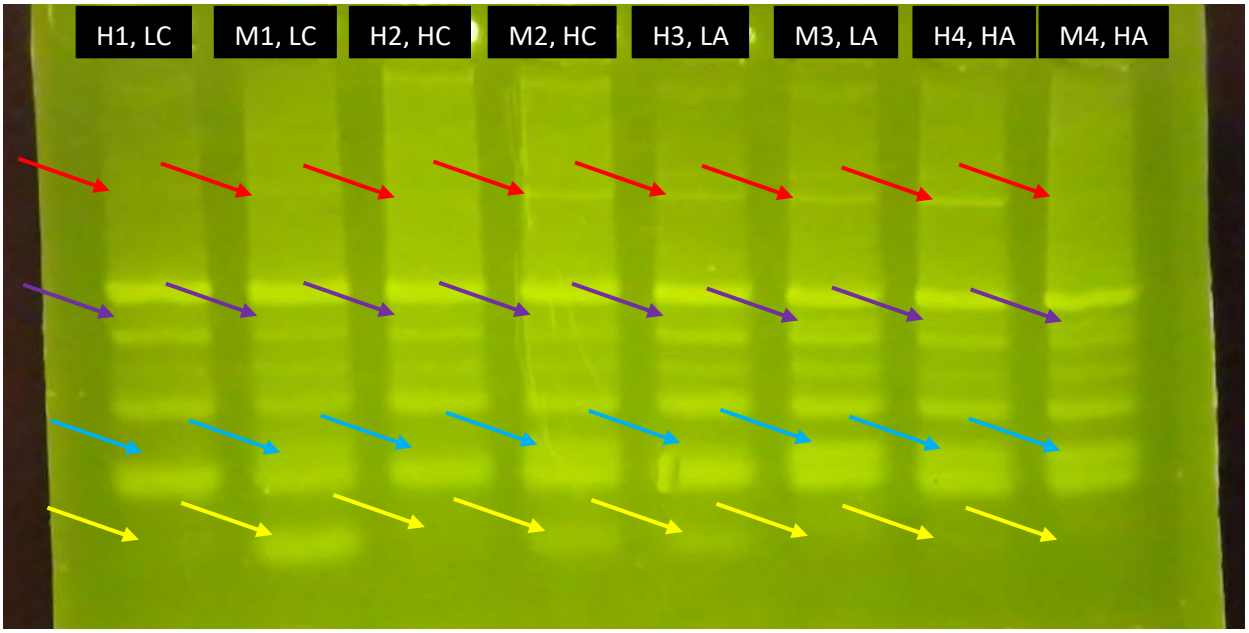
(Figure 7: Agarose gel of fragments amplified by PP_1. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



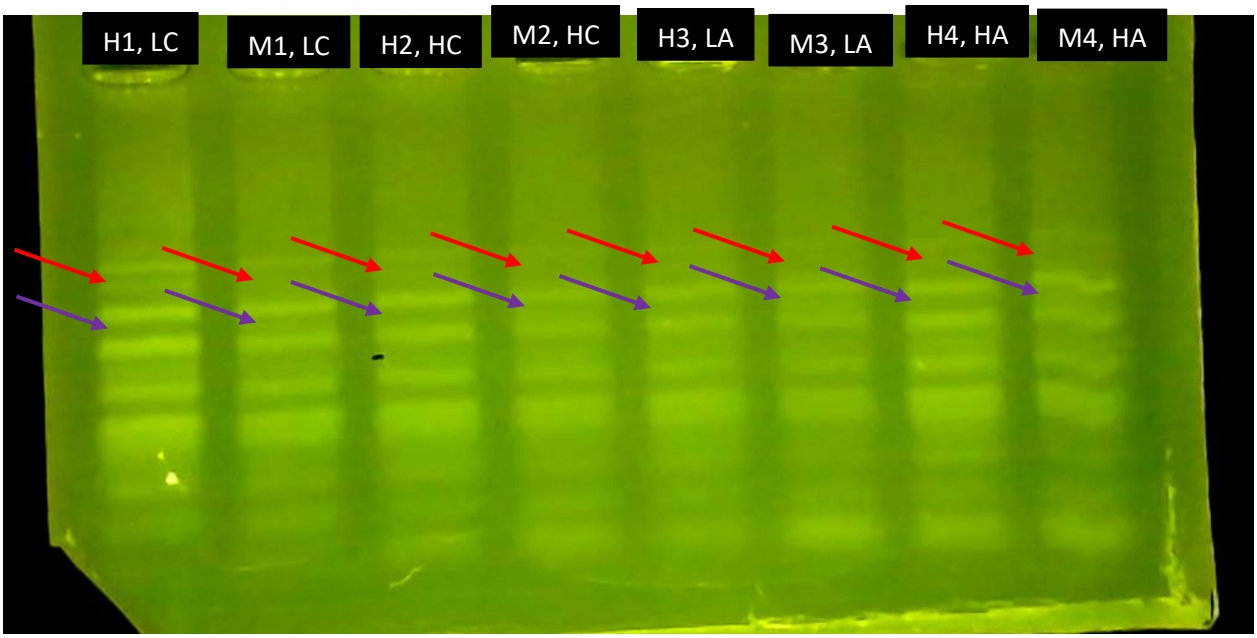
(Figure 8: Agarose gel of fragments amplified by PP_2. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



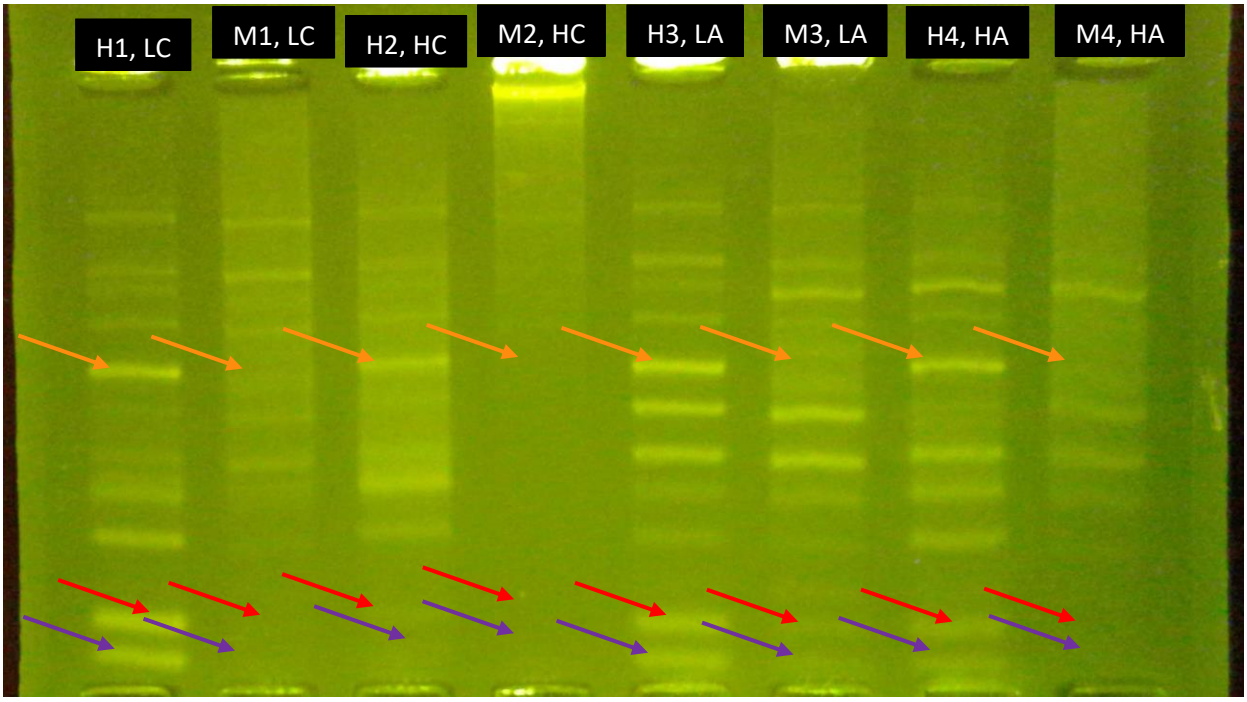
(Figure 9: Agarose gel of fragments amplified by PP_3. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



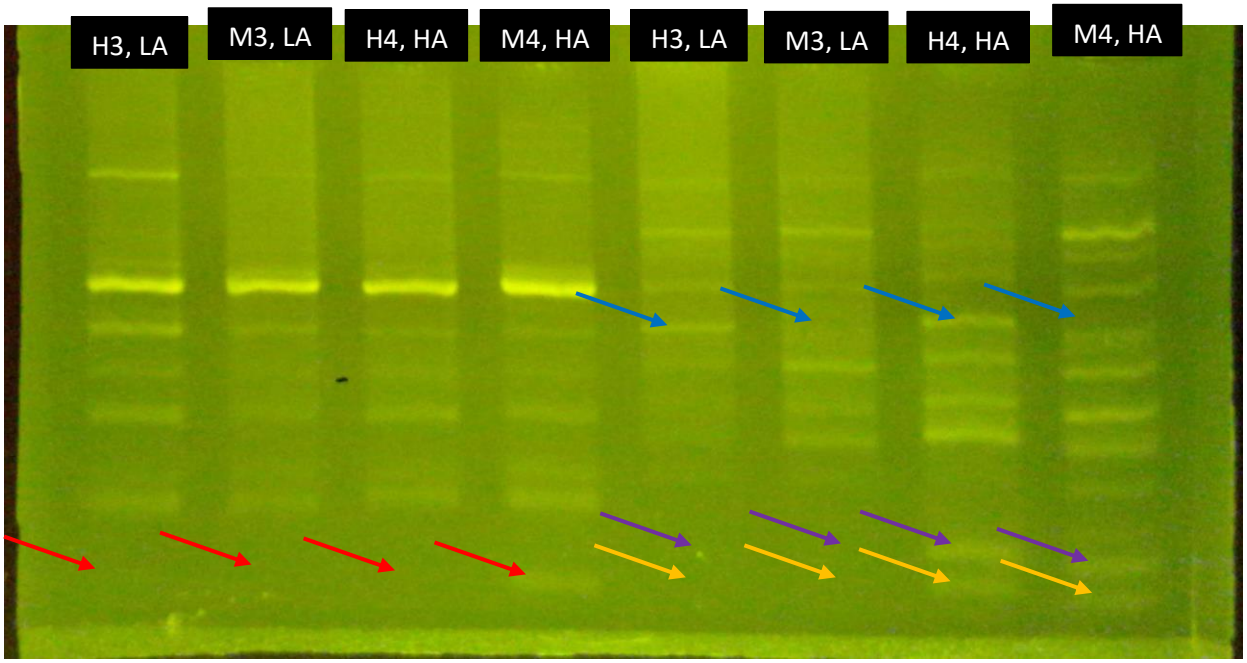
(Figure 10: Agarose gel of fragments amplified by PP_1. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



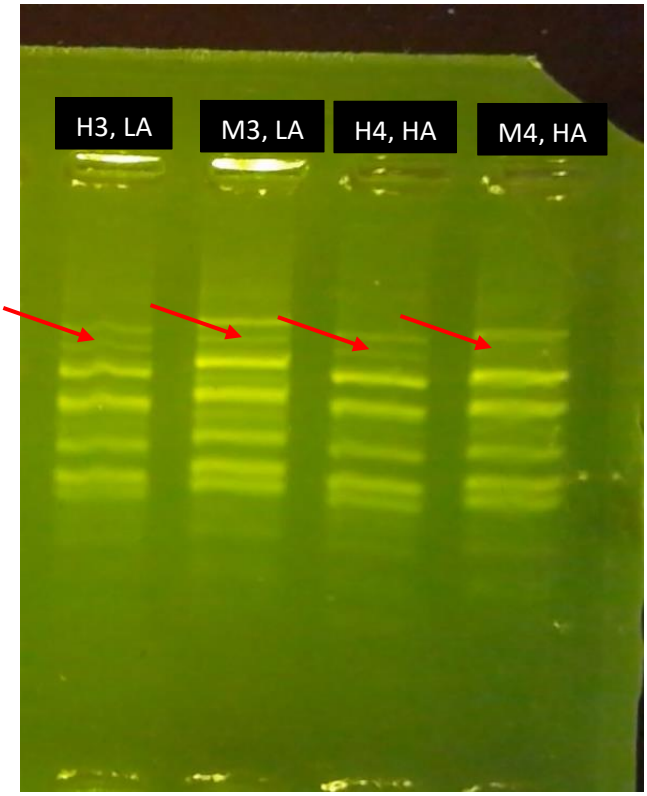
(Figure 11: Agarose gel of fragments amplified by PP_2. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



(Figure 12: Agarose gel of fragments amplified by PP_3. The red and purple arrows indicate differential methylation polymorphic fragments between treatments while the orange arrows indicate for hemi-methylated sites. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



(Figure 13: Agarose gel of fragments amplified by PP_1 and PP_3 where the first four columns contained fragments amplified by PP_1 while the last four columns contained fragments amplified by PP_3. The red, purple, and orange arrows indicate differential methylation states between treatments while the blue arrows indicate for hemi-methylated sites. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)



(Figure 14: Agarose gel of fragments amplified by PP_2. The arrows indicate differential methylation polymorphic fragments between treatments. Samples were labelled by the following: "H#" were digested with *HpaII*/*EcoRI*, "M#" were digested with *MspI*/*EcoRI*, "LC" were control groups grown in low CO_2, "HC" were control groups grown in high CO_2, "LA" were 5-azaC treated groups grown in in low CO_2, and "HA" were 5-azaC treated groups grown in high CO_2)

Discussion

Based on Figure 3 and Table 2, the highest proportions of fragments for each group were unmethylated while the lowest proportions of fragments were methylated for LC and LA and the lowest proportions of fragments were hemi-methylated for HC and HA. Also, the majority of the unmethylated fragments were not polymorphic since there were no changes in methylation states between most unmethylated fragments grown in low CO_2 when compared to their corresponding fragments that grew in high CO_2 (Figures 4- 14). Although, some fragments and their corresponding areas that are being pointed out, including the orange arrows in Figure 12 and the blue arrows in Figure 13, are an exception since all those sites were hemi-methylated instead of unmethylated between each treatment which means they are not polymorphic since there were no differences in methylation states. Therefore, the gels were scored by placing arrows next to hemi-methylated and methylated sites as well as next to their corresponding fragments and areas among the treatments because most of the polymorphic fragments were hemi-methylated or methylated (Figures 4- 14). Regarding the 5-azaC positive control, Table 2 shows evidence that 5-azaC caused demethylation since the proportions of unmethylated sites were greater in 5-azaC treated groups when compared to their corresponding control groups. In groups grown in low CO_2, the proportions of unmethylated sites were 69.74% and 79.93% for the control group and the 5-azaC treated group, respectively. Regarding groups grown in high CO_2, the proportions of unmethylated sites were 61.16% and 75.37% for the control group and the 5-azaC treated group, respectively.

As mentioned in the Material and methods section, both the appearance and the lack of fragments indicate different methylation states. Specifically focusing on the figures of the agarose gels (Figures 4 – 14), columns labelled with “H#” indicate that the site was cleaved by

HpaII/*EcoRI* while columns labelled with "M#" were cleaved by *MspI*/*EcoRI*. Therefore, if corresponding fragments are visible for both the H# and M# columns, then the site is unmethylated (corresponding meaning "H1" is coupled with "M1" for example). Also, if a fragment appears under the H# column, but there is no corresponding fragment under the M# column, then the fragment is hemi-methylated, likewise, if a fragment appears under the M# column, but there is no corresponding fragment under the H# column, then the fragment is methylated. On another note, based on Wang, *et al*. (2014), the absence of fragments may imply that a site was hypermethylated. Such a deduction can be made if corresponding areas in another treatment group contain fragments. The differential methylation states are identified in Tables 3, 4, and 5 where unmethylated, non-polymorphic fragments were excluded to easily compare differential methylation states between the agarose gels. Table 6 was developed using data from Tables 3, 4, and 5 where a total of twenty eight polymorphisms were identified; ten polymorphisms were identified between control groups grown in either low or high CO_2 while eighteen polymorphisms were identified between 5-azaC treated groups grown in either low or high CO_2. The highest proportion of polymorphisms were four polymorphisms that changed methylation states for 5-azaC treated sites, more specifically, where the methylation states changed from hemi-methylated to unmethylated, respectively (Table 6). This is reasonable since 5-azaC is a demethylation agent which is known to cause less methylation and more unmethylation to occur. Interestingly, three of those polymorphisms were exhibited on Figure 6 while the fourth polymorphism was exhibited on Figure 9 (Table 5). Such a result implies that differentially methylated polymorphisms are created via random methylation of the genome when plants are grown in different CO_2 concentrations. Similarly, the other polymorphisms appear to be randomly methylated so there is little to no consistency of methylation between the

fragments. For example, in Table 3, Figure 7, each fragment labelled with an arrow exhibited a polymorphism in either the control group or the 5-azaC treated group. This is not the case for Figure 9, referring to Table 5. For Figure 9, the red and yellow sections show no differential methylation, but the purple, orange, and blue sections show polymorphisms; the control groups showed differential methylation in the blue section while the 5-azaC treated groups exhibited differential methylation in the purple, orange, and blue sections.

Looking forward, the proportions of ambiguous sites were quite high (Table 2) but if this study was repeated, then improvements could be made so that more bands could be visible. More specifically, improving pipetting techniques could be beneficial. The faded bands were produced, possibly due to low DNA concentrations. This suggestion is reasonable because after DNA extraction, the resulting volume of the extracted DNA was higher than expected and produced low DNA concentrations. Also, some columns including "M2, HC" in Figure 4, "M1, LC" in Figure 12, and "H3, LA" in Figure 13 display an overall gradual fade in bands which may have been due to low DNA concentrations as well. Improving pipette techniques may prevent low DNA concentrations from being produced again. Furthermore, the column labelled, "M2, HC" shows a bright band at the top of the column, nearby the well. This suggests that the sample was not given adequate amounts of enzyme for digestion. More specifically, it was not digested properly during the *MspI* and/or the *EcoRI* digests because a new selective PCR sample was made but produced a similar bright band at the top of the column as well.

Improper handling of the agarose gels may have affected the bands as well. For example, faded and blurry bands were produced possibly due to abrupt movements while putting the cover on the gel tank because the movements may have caused the samples to seep out of the wells. Also, the agarose gels may have rested in the running buffer for an extended time which might

have caused the DNA to dissipate from the gels. On another note, the agarose may not have been swirled prior to pouring it into the casting gel; this could cause bands to smear since the gel matrix may not have been uniform. The column labelled, “H1, LC” in Figure 4 is one example of a smeared column. Also, after pouring the agarose into the casting tray, a pipette was used to remove air bubbles, but the agarose may have been disturbed too much since some bands, including the column labelled, “M4, HA” in Figure 6, appeared wavy.

Future research projects could refer to this study where differential methylation could be identified in other *A. thaliana* genotypes as well as in other plants, including both model and non-model organisms. DNA ladders were not applied to the gels in this study, but other studies may find it beneficial to add them to their gels since DNA ladders indicate that a gel is functional as well as provide fragment sizes which may be helpful for other studies. Also, DNA methylation techniques including bisulfate-sequencing and ELISA-based assay may identify differential methylation sites, compared to the sites identified by MS-AFLP.

Overall, differential methylation states were evident between ambient CO_2 concentrations and elevated CO_2 concentrations for both control and 5-azaC treated groups where a total of 28 polymorphisms were identified. In some cases, differential methylation was displayed by 5-azaC treated groups but was not seen in control groups within the same treatment. Such results imply that DNA treated with 5-azaC during plant development may affect how DNA is methylated if the organism is grown in different environmental conditions, specifically referring to differences in CO_2 concentrations. On another note, more demethylation was exhibited in 5-azaC treated groups when compared to control groups implying that this study is reliable.

Acknowledgements

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References

Alonso-Blanco, C. and Koornneef, M. 2000. Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in plant science 5*: 22-29.

Bossdorf, O., Richards, C. L., and Pigliucci, M. 2008. Epigenetics for ecologists. *Ecology Letters 11*: 106-115.

Bossdorf, O., Arcuri, D., Richards, C. L., and Pigliucci, M. 2010. Experimental alteration of DNA methylation affects phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana. Evolution Ecology 24*: 541–553.

Cervera, M. T., Ruiz-García, L., and Martínez-Zapater, J. M. 2002. Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation-sensitive AFLP markers. *Molecular Genetics and Genomics 268*: 543-552.

Clauss, M. J. and Aarssen, L. W. 1994. Patterns of reproductive effort in *Arabidopsis thaliana*: Confounding effects of size and developmental stage. *Ecoscience 1*: 153-159.

Dietterich, L. H., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A. D. B., Bloom, A. J., Carlisle, E., Fernando, N., Fitzgerald, G., Hasegawa, T., Holbrook, N. M., Nelson, R. L., Norton, R., Ottman, M. J., Raboy, V., Sakai, H., Sartor, K. A., Schwartz, J., Seveweera, S., Usui, Y., Yoshinaga, S., Myers, S. S. 2015. Impacts of elevated atmospheric CO_2 on nutrient content of important food crops. *Scientific Data 2*: 1-8.

Diévart, A and Clark, S. E. 2004. LRR-containing receptors regulating plant development and defense. *Development 131*: 251-261.

Feeley, K. J., Silman, M. R.,Bush, M. B., Farfan, W., Cabrera,K. G., Malhi, Y., Meir, P., Revilla, N. S., Quisiyupanqui, M. N. R., Saatchi, S. 2011. Upslope migration of Andean trees. *Journal of Biogeography 38*: 783-791.

Godfrey-Smith, P. Conditions for Evolution by Natural Selection. 2007. *The Journal of Philosophy 104*: 489-516.

Griffin, P. T., Niederhuth, C. E., Schmitz, R. J. 2016. A Comparative Analysis of 5-Azacytidine- and Zebularine-Induced DNA Demethylation. *G3: Genes, Genomes, Genetics 6*: 2773 – 2780.

He, X. J., Chen, T., Zhu, J. K. 2011. Regulation and function of DNA methylation in plants and animals. *Cell Research 21*: 442 – 465.

Hedenus, F., Wirsenius, S., and Johansson, D. J. A. 2013. The importance of reduced meat and dairy consumption for meeting stringent climate change targets. *Climatic Change 124*: 79-91.

IPCC. 2018. Summary for policy makers. *Climate change 2018: The Physical Science Basis.*

Jagadish, S. V. K., Bahuguna, R. N., Djanaguiraman, M., Gamuyao, R., Prasad, P. V. V., Craufurd, P. Q. 2016. Implications of High Temperatures and Elevated CO_2 on Flowering Time in Plants. *Frontiers in plant science 27*: 1-11.

Jump, A. S. and Peñuelas, J. 2015. Running to stand still: adaptation and the response of plants to rapid climate change. *Ecology Letters 8*: 1010-1020.

Nicotra, A. B., Atkin, O. K., Bonser, S. P., Davidson, A. M., Finnegan, E. J., Mathesius, U., Poot, P., Purugganan, M. D., Richards, C. L., Valladares, F., and van Kleunen, M. 2010. Plant phenotypic plasticity in a changing climate. *Trends in Plant Science 15*: 684- 692.

Pigliucci, M. 1998. Ecological and evolutionary genetics of *Arabidopsis*. *Trends in plant science 3*: 485 - 489.

Pigliucci, M. 2002. Ecology and Evolutionary Biology of *Arabidopsis*. *The Arabidopsis Book 1*: 1-20.

Pigliucci, M. 2008. Is evolvability evolvable. *Nature Reviews Genetics 9*: 75-82.

Richards, C. L., Alonso, C., Becker, C., Bossdorf, O., Bucher, E., Colomé-Tatché, M., Durka, W., Engelhardt, J., Gasper, B., Gogol-Döring, A., Grosse, I., van Gurp, T. P., Heer, K., Kronholm, I., Lampei, C., Latzel, V., Mirouze, M., Opgenoorth, L., Paun, O., Prohaska, S. J., Stadler, P. F., Trucchi, E., Ullrich, K., and Verhoeven, K. J. F. 2017. Ecological plant epigenetics: Evidence from model and non-model species, and the way forward. *Ecology Letters 20*: 1576-1590.

Richards, C. L., Schrey, A. W., and Pigliucci, M. 2012. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecology Letters 15*: 1016-1025.

Stenøien, H. K., Fenster, C. B., Tonteri, A., and Savolainen, O. 2005. Genetic variability in natural populations of *Arabidopsis thaliana* in northern Europe. *Molecular Ecology 14*: 137-148.

Teng, C., Wang, J., Chen, T., Wu, X., Wang, Y., and Lin, J. 2006. Elevated CO_2 induces physiological, biochemical and structural changes in leaves of *Arabidopsis thaliana*. *New Phytologist 172*: 92-103.

van der Kooi, C. J., Reich, M., Löw, M., De Kok, L. J., and Tausz, M. 2016. Growth and yield stimulation under elevated CO_2 and drought: A meta-analysis on crops. *Environmental and Experimental Botany 122*: 150-157.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research 23:* 4407–4414.

Wang, H., Dong, B., Jiang, J., Fang, W., Guan, Z., Liao, Y., Chen, S., Chen, F. 2014. Characterization of *in vitro* haploid and doubled haploid *Chrysanthemum morifolium* plants via unfertilized ovule culture for phenotypical traits and DNA methylation pattern. *Frontiers in Plant Science 5:* 1-10.

Wienkoop, S., Baginsky, S., Weckwerth, W. 2010. Arabidopsis thaliana as a model organism for plant proteome research. *Journal of Proteomics 73*: 2239 – 2248.

Zhang, M., Kimatu, J.N., Xu, K., Liu, B. 2010. DNA cytosine methylation in plant development. *Journal of Genetics and Genomics 37*: 1 -12.

Zhang, Y. Y., Fischer, M., Colot, V., and Bossdorf, O. 2012. Epigenetic variation creates potential for evolution of phenotypic plasticity. *New Phytologist 197*: 314-322.