The effect of temperature on the hypocotyl growth rate of wild-type and *cer10* mutant seeds of *Arabidopsis thaliana*

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Abstract

Arabidopsis thaliana is a flowering plant that has an important epicuticular wax layer on its stem. This wax layer serves as a barrier against environmental stressors, such as temperature, and is composed of lipids that are produced by the enzyme, enoyl-CoA reductase (ECR). However, a deletion mutation in the *cer* gene coding for the ECR enzyme results in decreased deposition of cuticular wax on the hypocotyl of *A. thaliana.* In light of this information, we investigated the effect of the *cer10* mutation and temperature on the hypocotyl growth rate of *A. thaliana* seeds. Our experiment compared the average hypocotyl growth rate of wild-type and *cer10* mutant *A. thaliana* seeds grown at 12°C, 17°C, and 30°C. The average hypocotyl growth rate of *cer10* mutant seeds is lower, but not statistically significantly lower than wild-type seeds (two-way ANOVA, p-value >0.05). In addition, we found that the hypocotyl growth rate increased, though not statistically significantly, in both wild-type and *cer10* mutant seeds as the temperature increased from 12°C to 17°C**.** It was found that the average hypocotyl growth rates were 0.060 +/- 0.011 µm/day and 0.069 +/- 0.034 µm/day at 12°C and 17°C respectively, for the wild-type seeds,. For the *cer10* mutant seeds, the average hypocotyl growth rate was 0.041 +/- 0.012 µm/day at 12°C and 0.046 +/- 0.014 µm/day at 17°C. The increased growth in the wild type versus the *cer10* mutant may be due to the survival benefits that normal cuticular wax production provides, as it allows the plant to retain water necessary for growth.

Introduction

Arabidopsis thaliana, also known as thale cress, is a small herbaceous plant that is part of the mustard (Brassicaeae) family (Koch *et al.* 2004). This weed has long been used as a tool for understanding the physiology and biochemistry of photoautotrophic organisms under defined growth conditions. The plant has a relatively short life cycle from seed germination to flowering of about 6 weeks, and its well-defined developmental features can lead researchers to predictable responses under different environmental conditions (Meinke et al. 1998).

A. thaliana has a small genome size of about 120 Mbp, which is relatively small for an autophototrophic higher plant (Meinke *et al.* 1998). In 2000, it became the first plant species to

have its whole genome sequenced (Koornneef and Meinke 2010). In recent years, research on *A. thaliana* has benefited greatly from the availability of many types of mutant lines, some of which have been selected for studies under extreme environmental conditions. Our study deals with the responses of the wild type and an *eceriferum* (*cer10*) mutant line to changing temperatures during the early stages of seed development.

Wild-type *A. thaliana* has an extensive epicuticular wax layer that gives it a distinct matte appearance (Zheng *et al.* 2005). This wax layer is composed of very-long-chain fatty acids (VLCFA), which are produced by the endoplasmic reticulum bound enoyl-CoA reductase (ECR; Koornneef *et al.* 1989). This epicuticular wax layer is important for survival because it acts as a protective barrier against environmental stresses (Jenks *et al.* 1995). The *cer10* mutant has a deletion in an important gene encoding for the production of ECR, and as a result, the synthesis of the wax layer is impaired in this mutant (Zheng *et al.* 2005). This mutation occurs when problems arise in ECR synthesis, affecting the chemical compositions of the epicuticular wax and, ultimately, reducing the presence of this wax by 60% (Zheng *et al.* 2005). The decreased load of epicuticular wax on *cer10* mutants alters the reflection of light from the plant surface giving the stem a darker green appearance (Koornneef *et al.* 1989).

The main objective of our experiment was to test the effects of temperature on the hypocotyl growth rate of the wild-type and *cer10* mutant seeds of *A. thaliana*. Since *A. thaliana* is easy to acquire and accepted as a model organism around the world, our results could potentially contribute to and improve future research by providing insight into ideal temperatures necessary for optimal plant growth. Therefore, our null and alternate hypotheses are:

H_{o1}: Increased temperature has no effect on the hypocotyl growth rate of *A. thaliana* seeds.

H_{a1}: Increased temperature has an effect on the hypocotyl growth rate of *A. thaliana* seeds.

H_{o2}: The presence of the *cer10* mutation has no effect on the hypocotyl growth rate of *A. thaliana* seeds.

H_{a2}: The presence of the *cer10* mutation has an effect on the hypocotyl growth rate of *A. thaliana* seeds.

H_{o3}: The effect of increased temperature on the hypocotyl growth rate of *A. thaliana* seeds is the same in both the wild-type and the *cer10* mutant.

H_{a3}: The effect of increased temperature on the hypocotyl growth rate of *A. thaliana* seeds is not the same in the wild-type and the *cer10* mutant.

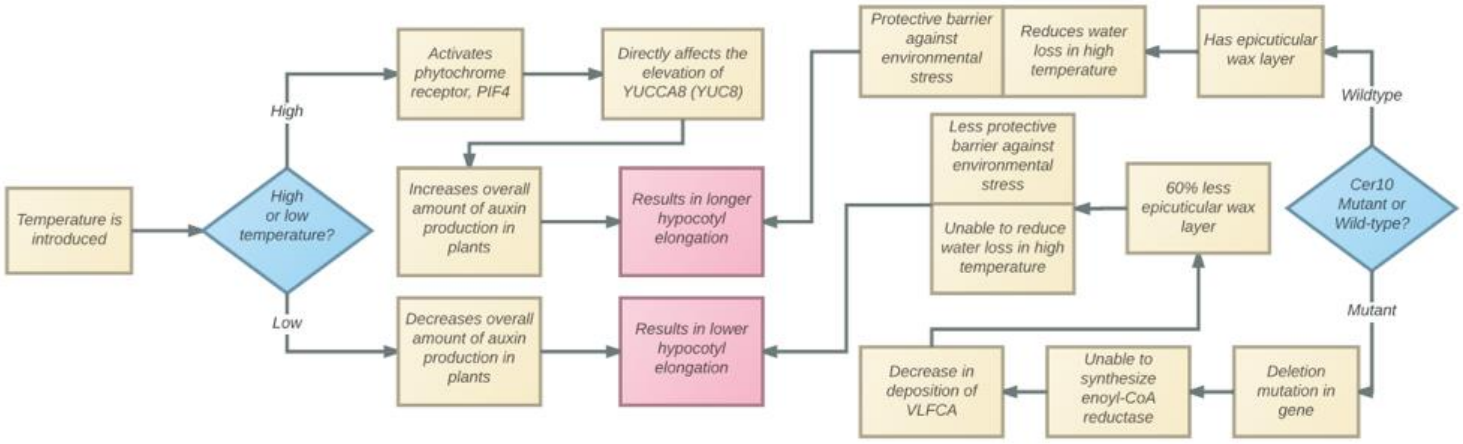

Figure 1. Flowchart depicting the different pathways that can lead to hypocotyl elongation in *A. thaliana*. The pathway on the left shows how high and low temperatures affect the production of the plant hormone, auxin. High temperatures increase the production of auxin, ultimately increasing hypocotyl elongation. The pathway on the right depicts how the *cer10* mutation can affect the plant's hypocotyl elongation. The *cer10* mutant has a decreased wax layer, resulting in decreased hypocotyl elongation.

We predicted that the *cer10* mutation would have an effect on the hypocotyl growth rate of the mutant seeds, and that temperature would have an effect on both the wild-type and mutant hypocotyl growth rates. Specifically, we also predicted that at higher temperatures, both the wild-type and *cer10* mutant seeds would have increased hypocotyl growth rates than at lower

temperatures, but the wild-type seeds would obtain higher hypocotyl growth rates than the *cer10* mutant seeds. Figure 1 (on the left) shows the different processes of how high and low temperatures could affect the increase or decrease of hypocotyl growth (Sun *et al.* 2012). According to Gray *et al.* (1998), when *A. thaliana* is exposed to a high temperature such as 29°C, its hypocotyl length increases more than it would if it had been grown at its more habitual temperature, such as 20°C. As shown in Figure 1, this is because high temperatures directly activate a type of phytochrome receptor, PIF4, in *A. thaliana* (Sun *et al.* 2012). This receptor is directly associated with YUCCA8 transcripts, which has been demonstrated to contribute to auxin production in plants (Sun *et al.* 2012). Auxin has an important role in plant development as it is an essential hormone for behavioural processes in a plant's life cycle, and is crucial for a plant's hypocotyl development (Leyser *et al.* 1993). Thus, the pathway in Figure 1 suggests that temperature is an important factor that regulates auxin to mediate this growth response as an increase in temperature through the activation of PIF4 would result in an increase in auxin levels, inducing hypocotyl elongation. Also shown in Figure 1, a plausible explanation as to why *cer10* mutants' hypocotyls may not grow as much as the hypocotyls of wild-type seeds: *cer10* mutants have 60% less epicuticular wax layer. The pathway on the right in Figure 1 shows that the *cer10* mutant seeds will retain less water when temperature is high. Therefore, the presence of a cuticular wax layer is critical to decrease water loss within *A. thaliana* in environments where water is limited (Shepherd and Griffiths 2006).

Methods

We placed *A. thaliana* seeds in incubators at 12°C, 17°C, and 30°C. , The 12°C and 30°C treatments were chosen as the extreme low temperature and extreme high temperature conditions, respectively. The optimal temperature range for growth of *A. thaliana* is 22-23 °C

(Rivero *et al.* 2014); however, the closest available temperature to this range was 17°C, which was therefore selected as the control

Each treatment consisted of 10 60-mm diameter Petri dishes lined with filter paper. Five of the 10 Petri dishes contained wild-type seeds and the remaining five contained *cer10* mutant seeds (Figure 2a, b). We used a random number generator to randomly order the dishes on the tray.

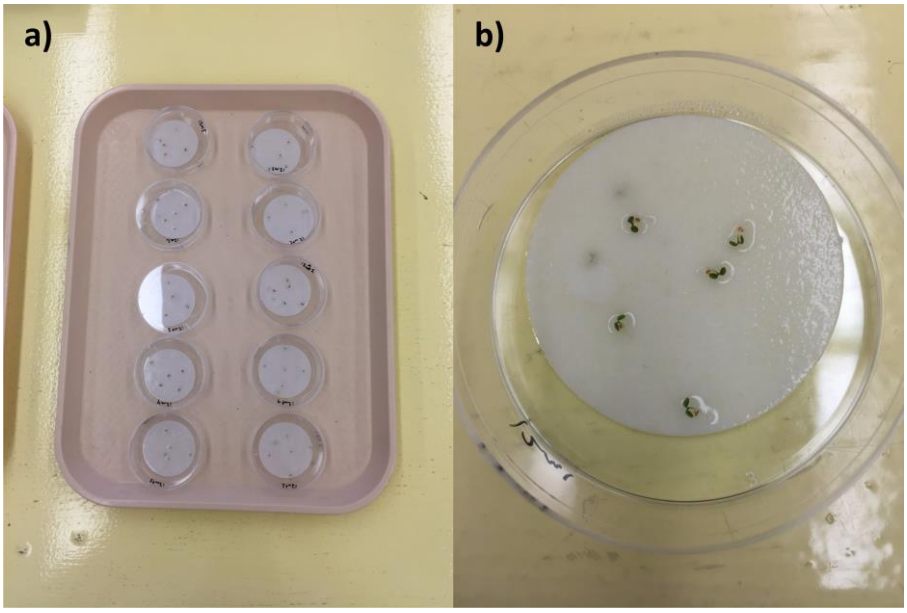

Figure 2. *Arabidopsis thaliana* seeds in Petri dishes taken on day 14 of the experiment. a) tray for incubator set at 12°C with five Petri dishes containing wild-type *A. thaliana* seeds and five Petri dishes containing *cer10* mutant *A. thaliana* seeds. Petri dishes arranged according to random number generation. b) Petri dish containing either five wild-type or five *cer10* mutant *A. thaliana* seeds.

Furthermore, it was vital to ensure other abiotic factors were kept constant such that they did not impact our results. To allow for constant light intensities for all three treatments, cheesecloth was placed over the trays in the 12^{o}C and 17^{o}C incubator to equate the light intensity with the 30^{o}C incubator's light intensity. Additionally, each time the trays were removed from the incubator, they were put back in the exact same spot. Lastly, the 30°C treatment received a larger volume of water in order to counteract the fast evaporation rate and to ensure seeds were

not under water deficit. Therefore, the treatments were watered based upon the level of filter paper saturation and at each watering session, the treatments were left with fully saturated filter paper.

Data were collected on days 3, 7, 10, and 14, by taking pictures of the 30 *A. thaliana* samples using a DinoXcope and a dissecting microscope. The pictures were analyzed using a program called ImageJ, which allowed us to see each seed in great detail, as shown in Figure 3. ImageJ was calibrated using instructions given by the program. The hypocotyl was measured from the base of the divergence of the two leaves, to the point at which the stem became the root, usually indicated by a change in colour (Figure 3a). The hypocotyl measurement is shown in Figure 3b, where the yellow, segmented line indicates the length of the hypocotyl. Seeds such as the one portrayed in Figure 3c were deemed to have no hypocotyl growth. Furthermore, qualitative observations were taken by looking at the seeds through the DinoXcope, as well as unmagnified. Notable observations included any visible change in hypocotyl length, viability of seeds, saturation of the filter paper, and colours of the seeds, leaves, and hypocotyls.

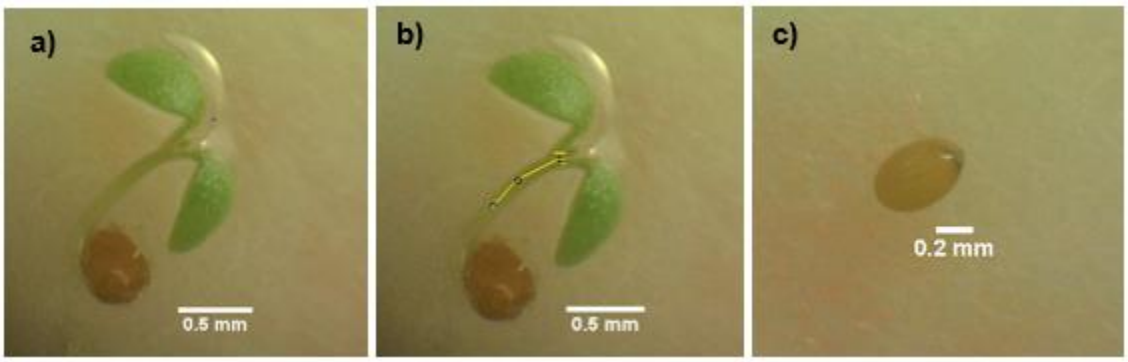

Figure 3. Pictures of wild-type *Arabidopsis thaliana* seeds taken on day 14 of data collection using a 1x objective lens magnification, shown on ImageJ. a) hypocotyl growth of a wild-type *A. thaliana* seed at 17°C. b) the same wild-type *A. thaliana* seed at 17°C with the segmented measuring tool showing a hypocotyl measurement. c) a wild-type *A. thaliana* seed at 30°C showing no signs of hypocotyl growth.

In order to calculate growth rate, we used the linear portion of the growth curves for the wild-type and *cer10* mutant *A. thaliana* seeds. Then, to determine the average change in length

of the hypocotyl, we found the difference in length between the third day of measurements (when we first saw hypocotyl growth) and the fifth (final) day of measurements. The average change in length of each replicate was then divided by the number of days between the measurements. This gave us the growth rate of each replicate in micrometers/day. Lastly[, , we took the mean] of the five average hypocotyl growth rates at each temperature, allowing us to obtain an overall average hypocotyl growth rate for wild-type and *cer10* mutant seeds at both temperatures. Using these values, we performed a two-way ANOVA statistical test to determine if any statistically significant differences existed among the means of our data. Finally, 95% confidence intervals were calculated to determine which values could have contributed to any significant differences in our treatments.

Results

Over the course of the 14 days of our experiment, it was consistently observed that the replicates exposed to the 12°C treatment did not deplete the water in their Petri dish (Figure 4). The replicates at 17°C required water to be added each time they were taken out of the incubator for watering or measurements, but did not require as much water as the replicates at 30°C, since the filter paper in the latter dishes tended to be completely dry each time we took them out of the incubator. It was also observed that the leaves of the replicates at 12°C (Figure 4), and at 17°C (not shown) looked healthy and green in colour, whereas there were no leaves evident for seeds in the 30°C treatment (Figure 3c). Wild-type and *cer10* mutant leaves looked similar in colour, but *cer10* mutant seeds in some Petri dishes looked darker than wild-type seeds, as seen in Figure 4.

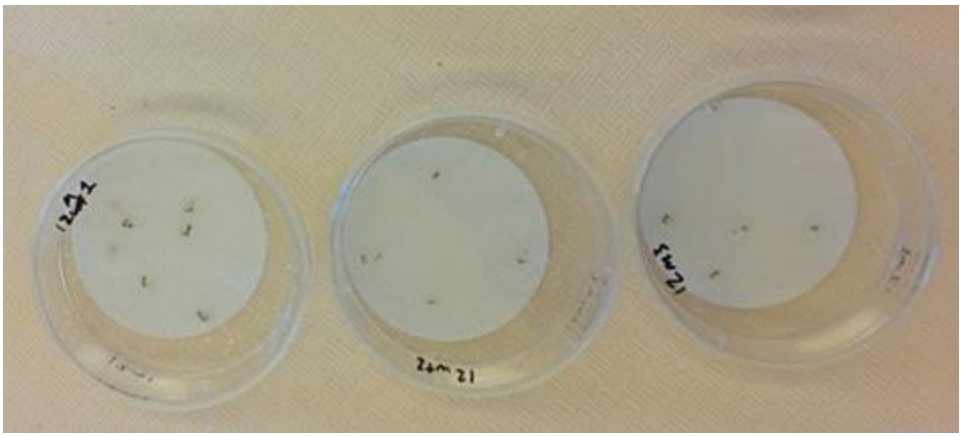

Figure 4. Three Petri dishes showing one wild-type replicate and two *cer10* mutant replicates exposed to a temperature of 12°C. Photo taken on day 14 of experiment.

As shown in Figure 5, the average hypocotyl growth rate for the wild-type seeds was 0.060 +/- 0.011 µm/day at 12°C and 0.069 +/- 0.034 µm/day at 17°C, whereas for the *cer10* mutant seeds, the average hypocotyl growth rate was 0.041 +/- 0.012 µm/day at 12°C and 0.046 +/- 0.014 µm/day at 17°C. There was no measurable growth of the wild-type and *cer10* mutant seeds at 30°C, and therefore, we did not include their measurements in our analysis. A two-way ANOVA was completed and *p*-values of 0.51, 0.053, and 0.89 were obtained for the first, second, and third hypotheses, respectively ($p > 0.05$). These values indicate that there were no statistically significant differences in the means of the treatments. To further support this, 95% confidence intervals were calculated, and as shown in Figure 5, the intervals overlap.

Despite the absence of statistically significant differences, a small variation exists between the average hypocotyl growth rate of the wild-type and *cer10* mutant seeds at each temperature, as the average hypocotyl growth rate of the wild-type seeds was 1.5 times larger than the average hypocotyl growth rate of the *cer10* mutant at both 12°C and 17°C. Wild-type seeds have a higher average hypocotyl growth rate than the *cer10* mutant seeds at each temperature, but the average hypocotyl growth rate increases for both types of seeds at 17°C compared to at 12°C (Figure 5).

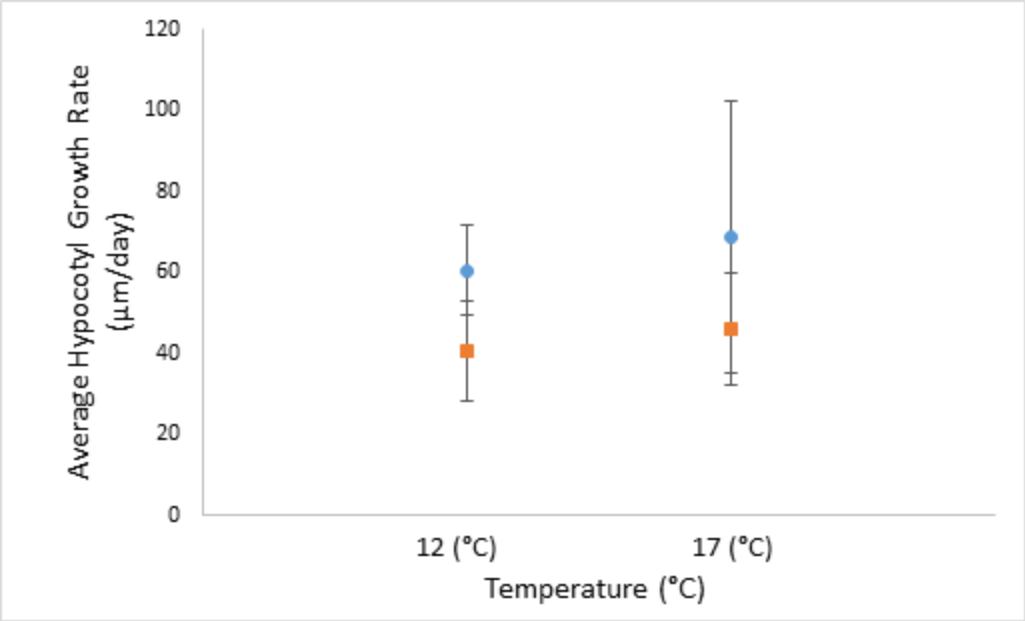

Figure 5. Average hypocotyl growth rate of wild-type (blue circles) and *cer10* mutant (orange squares) seeds of *A. thaliana* at 12°C and 17°C. Points represent the means of the average hypocotyl growth in µm/day. Error bars represent the 95% confidence intervals. Two-way ANOVA completed and *p*-values = 0.51, 0.053, and 0.89 for the first, second, and third hypotheses, respectively. n=5 for each treatment.

Discussion

We failed to reject all three of our null hypotheses. This was determined by results of a two-way ANOVA, as no statistically significant differences were found. There were no statistically significant differences between the average hypocotyl growth rate of the seeds at different temperatures ($p = 0.51$); the presence of the *cer10* mutation was not found to be statistically significant ($p = 0.053$); and finally, no statistically significant differences were found for the effect of temperature on the average hypocotyl growth rate between the wild-type and *cer10* mutant seeds ($p = 0.89$). Although the *p*-value of 0.053 was not statistically significant, but the value suggests that the *cer10* mutation could have a potential effect on the hypocotyl growth rate, but further studies would need to be done to see if this is the case. Furthermore, the results obtained do support our predictions, as the *cer10* mutation had an effect on hypocotyl growth rate, temperature had an effect on the hypocotyl growth rates of both types of seeds, and wild-type seeds had higher hypocotyl growth rates than the *cer10* mutant seeds. While there were no

statistically significant differences in our results, it was evident that the average hypocotyl growth rate increased for each type of seed as the temperature increased from 12°C to 17°C. In addition, the average hypocotyl growth rate of the wild-type seeds was greater than the average hypocotyl growth rate of the *cer10* mutant seeds.

Gray *et al.* (1998) examined the effect of temperature on the elongation of the hypocotyl and found that higher temperatures promoted a higher production of auxin. In our experiment, we expected to see greater differences in the growth rates between temperatures, as the pathway in Figure 1 explains how high temperatures lead to the increased production of auxin, which has a role in lengthening the hypocotyl. Our lack of statistically significant results is not consistent with the findings of Gray *et al.* (1998) and this could be due to the difference in the experimental condition of the experiments. Gray *et al.* (1998) had an experimental setup where the test plants were grown under continuous light, whereas our experimental setup utilized a day/night cycle of 16 hours of light and 8 hours of darkness. This difference in light conditions may have impacted the results.

Cuticular waxes of plants have been found to be important to the plant's response to external environmental factors (Shepherd and Griffiths 2006); therefore, since the *cer10* mutant of *A. thaliana* has a decreased wax layer, a significant difference was expected in the hypocotyl growth rate between the wild-type and mutant when exposed to different temperatures. Jenks *et al.* (1995) found that the wax layers of *A. thaliana* play an important role in the plant's survivability. While the average hypocotyl growth rates between the *cer10* mutant and the wild-type seeds were not statistically different, the average hypocotyl growth rate of the wild type was higher than the average hypocotyl growth rate of the *cer10* mutant. Shepherd and Griffiths (2006) found that external waxes played a key role in limiting water loss by decreasing the

plant's permeability when under osmotic stress. In our experimental setup, we aimed to control abiotic factors to provide the best growth environment, and by doing this, we also limited the role that the waxes play in the plant's ability to survive. For the 12^{o}C and 17^{o}C treatment, it was found that the filter paper never dried out between watering sessions, indicating that the wax layer did not have as much of an effect on the plant's ability to survive the experimental conditions. This would explain why the even though wild type had a higher average hypocotyl growth rate compared to the mutant these results were not statistically significant.

The replicates in the 30^{o}C treatment were found to not have any measurable growth during the experimental period. The cause for this lack of growth is believed to be dehydration of the seeds, as the temperature may have been too high for growth to occur. The water in each Petri dish evaporated quickly in this treatment, leading to dried out filter papers and the removal of the seeds' main source of nutrients. The filter papers were found to be dry at the beginning of multiple watering periods, meaning that dehydration was a persistent problem for the 30^{o}C treatment throughout the experiment. It was only during the final few days of observation that root growth was observed, but there was no significant hypocotyl growth by the end of the testing period.

Rivero *et al.* (2014) discuss how *Arabidopsis* plants usually germinate after 3-5 days under continuous light. This time frame falls within our experimental period of 14 days, but due to our measurements being taken every three or four days, we did not obtain the hypocotyl growth data for the initial growth. We found that there was an initial, rapid sprouting of the seeds as they grew over half of their total growth amount between two measurement days. After the initial growth, it was found that the hypocotyl grew more slowly.

A challenge we came across while performing the experiment was the subjectivity in determining where the hypocotyl deviated from the root of the organism. It was often difficult to ascertain where the hypocotyl started due to the very subtle change in colour that indicates where the hypocotyl ends and the root begins Gray *et al.* (1998) used more precise methods and equipment to consistently measure the hypocotyl length, such as shadow projections with a Simmon Omega variable condenser. In addition, the hypocotyl measurements were completed by different group members, which could have caused increased variation in the measurements.

The control temperature chosen was 17^{o}C and was outside of the plant's optimal temperature range of 22-23 ^{o} C. In addition, the low temperature was 12^{o}C, narrowing the range of temperatures tested in the experiment. The close nature of the test temperatures may have led to the similar results in the two temperatures. A wider range in temperatures could accentuate the differences and provide a better understanding of the effect of temperature on the growth of *A. thaliana*.

Conclusion

According to our results, we failed to reject all three of our null hypotheses. However, we found that when temperature increased from 12°C to 17°C, the average hypocotyl growth rate did as well. While there were no statistically significant differences between the average hypocotyl growth rates of the *cer10* mutant and wild-type *A. thaliana* seeds, the wild-type seeds did have a higher mean hypocotyl growth rate compared to the *cer10* mutant seeds. Lastly, we discovered that temperature and the presence or absence of the *cer10* mutation did not have statistically significant effects on the mean hypocotyl growth rate.

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