

# Effect of abscisic acid on the germination of wild-type and *cer10* mutant *Arabidopsis thaliana* seeds

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## Abstract

A mutation of the *cer10* gene expressed in *Arabidopsis thaliana* results in decreased cuticle wax accumulation, which in turn inhibits the growth of the plant. Abscisic acid (ABA) promotes the accumulation of wax by using functional proteins and also promotes growth of the plant. This study examines the effect of ABA on the growth rate of the hypocotyl of the mutant and wild-type *A. thaliana* seeds. Five different treatments of ABA (0 $\mu$ M, 2 $\mu$ M, 20 $\mu$ M, 100 $\mu$ M, and 200 $\mu$ M) were applied to both wild-type and mutant seeds for a period of 11 days. The difference in growth of the hypocotyls of wild-type and mutant seeds yielded a p-value of 0.00024 using a two-way ANOVA test, indicating that the expression of the *cer10* gene has a significant effect on hypocotyl growth in wild-type and mutant seeds over a period of 11 days. The impact of the ABA treatments on *A. thaliana* resulted in a p-value of  $2.74 \times 10^{-14}$ , which indicated that the ABA treatments did, in fact, affect hypocotyl growth. Comparing the germination rates with and without ABA within wild-type and mutant seeds yielded a p-value of  $3.89 \times 10^{-6}$ . This result indicates that increasing concentrations of ABA led to decreasing hypocotyl growth for the seeds.

## Introduction

A mutation of the *cer10* gene expressed in *Arabidopsis thaliana* changes the cuticular profile of the stem and leaves of the organism. Instead of having many long alkanes in the cuticle as present in the wild-type, the *cer10* mutation has an increased number of reduced alkanes and alcohols present, which decreases the overall accumulation of wax in the cuticle (Rashotte, Jenks & Feldman 2001). The expression of this gene is necessary for healthy growth of *A. thaliana*. Comparing the wild-type and *cer10* mutant *A. thaliana* phenotypes, the mutant was observed to have the following abnormalities: leaves that were more frail and fewer in number, shorter and weaker stalks, decreased cuticle wax accumulation, along with shorter and crooked stamen filaments (Zheng et al. 2005). We studied the relationship between the hypocotyl growth of the *cer10* mutant and wild-type seeds of *A. thaliana* as well as the effects of the hormone abscisic acid (ABA) on these seeds.

ABA is a hormone that increases cuticular wax production by inducing the transcription of other wax associated genes within the organism (Bernard et al. 2013). The accumulation of protectants such as small hydrophilic proteins and sugars are induced by ABA, which also activates detoxifying mechanisms that increase the organism's stress tolerance by better regulating the transport of ions, to maintain homeostasis (Finkelstein 2013). The same study conducted by Finkelstein (2013) also found that elevated levels of ABA promoted root growth and inhibited shoot growth of the organism, leading to positive hydrotropism, meaning the roots were better able to find moisture allowing more growth of the plant overall. The overall impacts of the addition of ABA can also be seen in Figure 1, where the addition helps the plant grow through increasing the accumulation of wax, and a lack of ABA has no effect on growth.

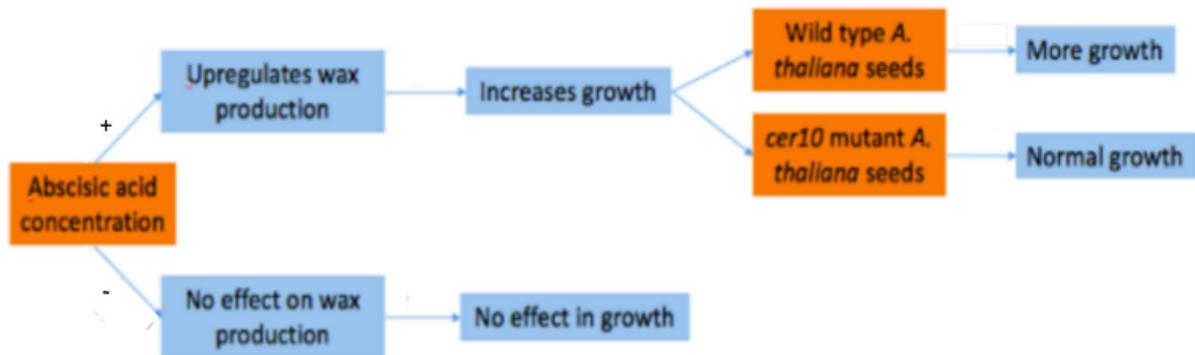
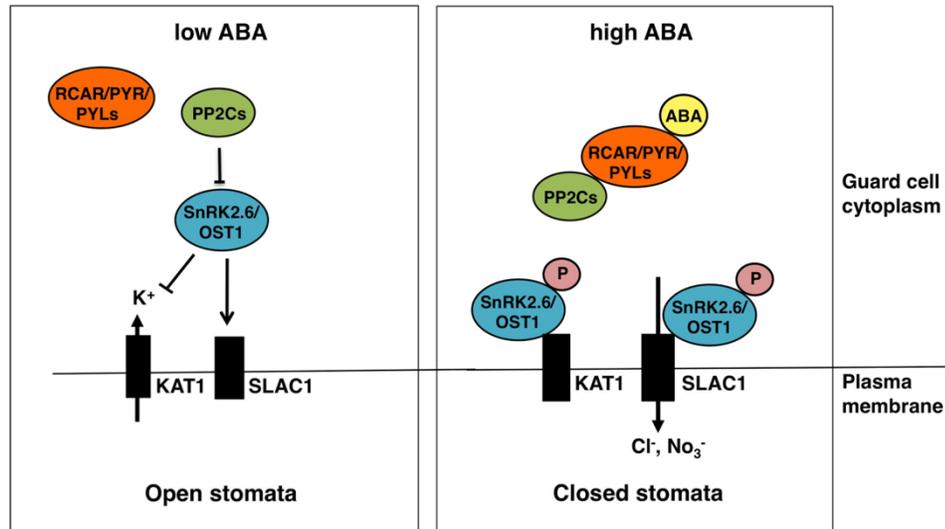


Figure 1. A model representing our prediction for the changes in the relative hypocotyl growth rates between the mutant and wild type *A. thaliana*, upon addition of ABA.

ABA works by regulating the gene expression for the SnRK2 III subfamily of SnRK proteins in response to stressful conditions (Fernando & Schroeder 2016). These SnRK2 kinases are important for the regulation of the stomatal closure in response to stress, inhibiting seedling growth in response to stress and playing a crucial role in osmotic stress signaling and stress tolerance for *A. thaliana* (Fernando & Schroeder 2016). A model of this mechanism can be seen on Figure 2.



(Fernando & Schroeder, 2016)

Figure 2. A model comparing the effects of the presence and absence of ABA in *A. thaliana*. A high concentration of ABA shows the phosphorylation of the SnRK2 proteins leading to the closure of the stomata when introduced to salt in the environment.

We will be testing the following three hypotheses:

Hypothesis 1

H<sub>01</sub>: ABA concentration has no effect on hypocotyl growth of *A. thaliana* seeds.

H<sub>a1</sub>: ABA concentration has an effect on hypocotyl growth of *A. thaliana* seeds.

Hypothesis 2

H<sub>02</sub>: Presence of the *cer10* mutation has no effect on hypocotyl growth of *A. thaliana* seeds.

H<sub>a2</sub>: Presence of the *cer10* mutation has an effect on hypocotyl growth of *A. thaliana* seeds.

Hypothesis 3

H<sub>03</sub>: The effect of ABA concentration on hypocotyl growth of *A. thaliana* is the same in wild-type and *cer10* mutant seeds.

H<sub>a3</sub>: The effect of ABA concentration on hypocotyl growth of *A. thaliana* is not the same in the wild-type and *cer10* mutant seeds.

From the literature we understand that the application of ABA can help stimulate the growth of the plant overall, however we were curious to find how the application of ABA would

affect the hypocotyl growth of the seeds. According to Zheng et al. (2005), the *cer10* mutant of *A. thaliana* showed abnormal growth relative to the wild-type strain, and therefore, we predicted that the hypocotyl of wild-type *A. thaliana* seeds will grow faster than that of the mutant *A. thaliana* seeds. Additionally, as the phenotype from the mutation in the *cer10* gene and the effects of ABA work antagonistically, we predicted that adding ABA in higher concentrations to the mutant *A. thaliana* seeds will compensate for their delayed hypocotyl growth, thereby accelerating the mutant hypocotyl growth to a hypocotyl growth rate similar to that of the wild type *A. thaliana*.

## **Methods**

This experiment was designed to investigate the effect of different ABA treatments on the germination of wild-type and *cer10* mutant *A. thaliana* seeds over the course of 11 days, two of which were for the initial preparation. Five treatments were used for both wild-type and *cer10* mutant seeds. Treatment one was 0 $\mu$ M ABA, treatment two was 2 $\mu$ M of ABA, treatment three was 20 $\mu$ M of ABA, treatment four was 100 $\mu$ M of ABA, and treatment five was 200 $\mu$ M of ABA. Treatments two, three and four were all diluted from stock 200 $\mu$ M of ABA and stock tap water. Treatments were then stored in labelled bottles at room temperature.

Wild-type and *cer10* mutant seeds were separated into two trays, as shown in Figure 3. Each tray consisted of five treatments, as mentioned above. We placed three replicates in each treatment. Each replicate consisted of a 60-mm petri dish, filter paper and 10 seeds of either wild-type or mutant *A. thaliana*.



Figure 3. Setup of wild type and mutant seeds. The trays were separated into either wild-type (right) or mutant (left) seeds. Each tray had five treatments – 0 $\mu$ M, 2 $\mu$ M, 20 $\mu$ M, 100 $\mu$ M, and 200 $\mu$ M ABA. Each treatment had three replicates labelled with seed type, treatment and order of position.

A corresponding treatment of 1000 $\mu$ L was added for each replicate on day 1. We added treatment ABA every day; the volume of treatment ABA added was dependent on the dryness level of the filter paper. We added 500 $\mu$ L of treatment ABA for replicates with very dry filter paper, whereas a range of 150 $\mu$ L to 300 $\mu$ L of treatment ABA was added to replicates containing filter paper that was moist but not saturated.

We placed all treatments of *A. thaliana* in a growth chamber with constant humidity and temperature set to 20°C. The growth chamber was set to display a cycle of 16 hours of light and 8 hours of darkness to simulate natural lighting. From day 1 to day 8, the wild-type and *cer10* mutant seeds trays were positioned vertically with one tray towards the inner portion and the other towards the door of the growth chamber (Figure 4a). A slight difference in light intensity between the two positions in the growth chamber was noticed and therefore, we alternated the position of the trays every day. In addition, the humidity of the growth chamber was increased because dry filter papers were observed at similar regions. On day 9, both trays were positioned near the door of the growth chamber as alternating vertical position did not solve the issue of some of the filter paper drying (Figure 4b).



Figure 4. (a) Alternating position of wild-type and mutant seeds tray from day 1 to day 8; (b) Change in position of wild-type and mutant seed trays to both positioned near the door at day 9. The middle tray wrapped with a white cloth was another group's experiment which we were unable to reposition.

As seen in Figure 5, we set up a dissecting microscope inserted with a DinoXcope eyepiece which was then linked to a laptop for taking photos of the seeds of all replicates. We used image J program to measure the length of the hypocotyl, which was the axis of the seedling below its first leaf. This length was measured for all seeds every second day of the experiment after preparation.

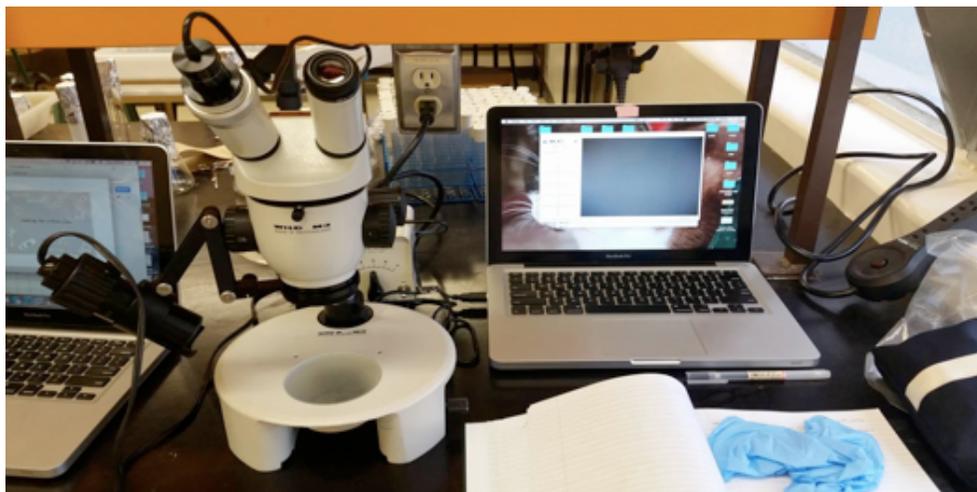


Figure 5. Setup of equipment to measure the length of the hypocotyl, including the length of the seed. A dissecting microscope was inserted with a DinoXcope eyepiece which was linked to a laptop to take photos.

For the results, we averaged the lengths of the replicates per treatment, which was then used to calculate the hypocotyl growth rate. We used a two-way ANOVA statistical test to

compare the hypocotyl growth rate between wild-type and *cer10* mutant *A. thaliana*. Via the statistical test, the p-values for the corresponding hypotheses were determined and the 95% confidence intervals were calculated.

## Results

Both the wild-type and mutant *A. thaliana* had the greatest hypocotyl growth rate when they were exposed to 0 $\mu$ M ABA concentration treatment (Figure 6). The largest difference in hypocotyl growth was between the wild-type and *cer10* mutant seeds in the control 0 $\mu$ M ABA treatment. The *cer10* mutant had the most hypocotyl growth of  $1.614 \pm 0.244$ mm/day compared to the wild type which had  $0.831 \pm 0.160$  mm/day. When the ABA treatments were added to each replicate whether wild-type or *cer10* mutant, each replicate responded similarly in average hypocotyl growth with very minimal growth regardless of the ABA treatment concentration.

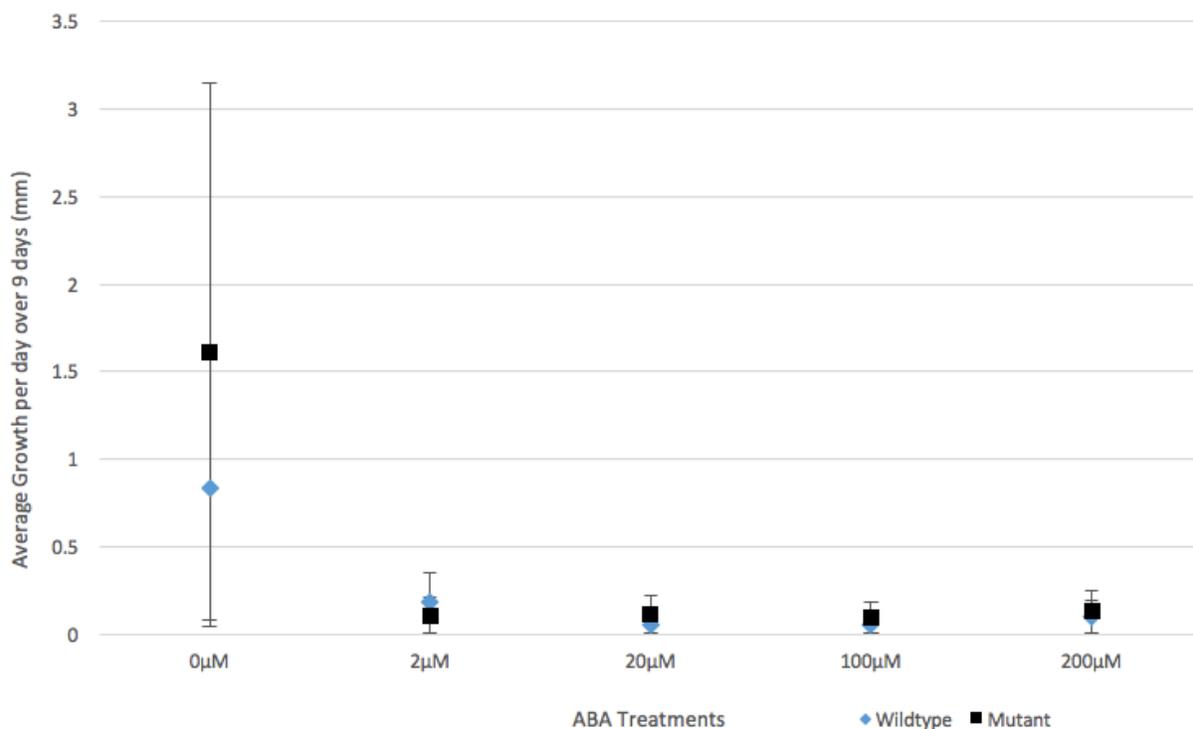


Figure 6. The relationship between the average hypocotyl growth over 9 days between wild-type and *cer10* mutant *A. thaliana* seeds when exposed to treatments of 0 $\mu$ M, 2 $\mu$ M, 20 $\mu$ M, 100 $\mu$ M and 200 $\mu$ M ABA. The p-value for  $H_1$  was 0.00024,  $H_2$  was  $2.74 \times 10^{-14}$  and  $H_3$  was  $3.89 \times 10^{-6}$ . Error bars represent 95% confidence intervals, n=3.

From the two-way ANOVA test: the effect of ABA concentration treatment on hypocotyl growth,  $p = 0.00024$ , the effect of the mutation,  $p = 2.74 \times 10^{-14}$ , and the comparison of the effect of the treatment on wild type and mutant,  $p = 3.89 \times 10^{-6}$ .

## **Discussion**

From the results, we reject the first null hypothesis and provide support for the alternate hypothesis that ABA concentration has an effect on hypocotyl growth. The p-value was  $2.74 \times 10^{-14}$ , which is less than the alpha value of 0.05. We also reject the second null hypothesis and provide support for the alternate hypothesis that the presence of the *cer10* mutation has an effect on hypocotyl growth. The p-value was 0.00024, which is less than the alpha value of 0.05. Lastly, we reject the third null hypothesis and provide support for the alternate hypothesis that the effect of ABA concentration on hypocotyl growth is not the same in the wild-type and *cer10* mutant seeds. The p-value was  $3.89 \times 10^{-6}$  which is less than the alpha value of 0.05.

Initially, we predicted that the addition of ABA to *cer10* mutant *A. thaliana* seeds would improve the hypocotyl growth rate because Bernard and Joubes (2013) observed that the addition of ABA to *A. thaliana* seedlings improves plant growth. However, upon further research, we discovered that ABA concentration does not improve seed germination, but in fact it reduces it (Ghassemian et al. 2000; Zhao 2012). Previous work done by Planes et al. (2014) showed that the addition of ABA to *A. thaliana* seeds resulted in germination inhibition, while work done by Ghassemian et al. (2000) found that the addition of ABA to *A. thaliana* seedling roots resulted in an increase in growth. These findings contrast our previous thoughts on the effect of ABA which led to our prediction that ABA would increase hypocotyl growth in seeds and growth in seedlings.

We observed a significant decrease in average germination (Figure 6) upon the addition of ABA treatment. Our results are consistent with previous work done by Karssen et al. (1982) who observed that seeds without ABA treatment germinated, while seeds with ABA treatment germinated at a slower rate. Slow hypocotyl growth of ABA treated seeds may be due to ABA causing a delay in the emergence of the hypocotyl from the seed (Jia et al. 2012). The delayed growth of the hypocotyl occurs due to an increase of chemical interactions between molecules of the cell wall induced by ABA, which increases the physical resistance of the seed, and therefore, prevents the emergence of the hypocotyl (Jia et al. 2012). Another reason we observed a decrease of average germination could be due to an increase in cytosolic acidification. Planes et al. (2014) observed that the addition of ABA treatment inhibits the activity of the enzyme ATPase. The inhibition of ATPase allows for the accumulation of protons in the cytosol which inhibits germination (Planes et al 2014).

In this study, the *cer10* mutation is observed to have an effect on hypocotyl growth. Usually, the *cer10* mutant seeds exhibit an impaired growth of the hypocotyl due to a reduction in the expression of the enoyl-CoA reductase (ECR) gene which is important for the synthesis of triacylglycerols (TAGs) (Beaudoin et al. 2009). TAGs are important for the germination of *A. thaliana* seeds because they are used for energy before the seedlings are able to fully sustain themselves photosynthetically (Shrestha et al. 2016). However, we observed greater hypocotyl growth in the *cer10* mutant seeds in 0mM ABA treatment compared to the wild-type seeds in 0mM ABA treatment (Figure 6). A possible explanation for this could be that several filter papers repeatedly dried which resulted in seeds shriveling, which ultimately impacted the normal growth of the hypocotyl.

Many errors were made during the experiment, both human and systemic. Despite being in the same growth chamber, the amount of light each replicate was exposed to was not uniform. We noticed that the tray that was placed on the inner side of the growth chamber always had dry filter papers at the upper right region. Although the humidity of the chamber was increased and trays were repositioned, dried filter papers were still observed and may have caused a disruption in the germination of the seeds.

Our experiment was forced to end due to mold contamination in two mutant control replicates. Nine days were not enough for sufficient results in our experiment because growth of seeds from ABA treatments was observed at approximately day 9. If we continued the experiment for a longer period of time, we may have been able to observe significant growth of the seeds treated with ABA, which we believe may have resulted in a different conclusion. Furthermore, the mold suggested that we should have done a sterile experiment next time to prevent contamination. On the other hand, contamination may have been due to human error, either through touching the pipette tip on unsterile surfaces or leaving the dishes exposed to the external environment too long when adding treatments.

## **Conclusion**

Initially, we predicted that the addition of ABA would impact hypocotyl growth of both types of the *A. thaliana* seeds and this prediction was confirmed. With respect to our second hypothesis, we concurred with our initial prediction and the *cer10* mutation was determined to have an effect on hypocotyl growth for both wild type and *cer10* mutant *A. thaliana* seeds. Additionally, it was predicted that the ABA treatment would affect the hypocotyl growth rate for the *cer10* mutated seeds and wild-type seeds differently; this prediction was also supported by our data. In contrast to our initial predictions however, it was found that an addition of ABA

treatment led to a decrease in germination and hypocotyl growth for both types of seeds. The statistical analysis of the data suggested that although the ABA treatment impacted the hypocotyl growth of the seeds; the ABA treatments further delayed the hypocotyl growth of the seeds rather than accelerating the growth.

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### **Literature Cited**

- Beaudoin, F, Wu, X, Li, F, Haslam, RP, Markham, JE, Zheng, H, Napier, JA & Kunst, L 2009, Functional characterization of the *Arabidopsis*  $\beta$ -ketoacyl-coenzyme a reductase candidates of the fatty acid elongase, *Plant Physiology*, vol. 150, no. 3, pp. 1174-1191.
- Bernard, A & Joubes, J 2013, *Arabidopsis* cuticular waxes: Advances in synthesis, export and regulation, *Progress in Lipid Research*, vol. 5, no. 1, pp. 110-129.
- Finkelstein, R 2013, Abscisic acid synthesis and response, *The Arabidopsis Book*, vol. 11, no. 166, pp. 1-36.
- Fernando, D & Schroeder, F 2016, *Role of ABA in Arabidopsis Salt, Drought, and Desiccation Tolerance*, Intech, Winnipeg.
- Ghassemian, M, Nambara, E, Cutler, S, Kawaide, H, Kamiya, Y & McCourt, P 2000, Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*, *The Plant Cell*, vol. 12, no. 7, pp. 1117-1126.
- Jia, A, Wu, Q, Ye, N, Liu, R, Xu, W, Zhi, H, Rahman, RB, Xia, Y & Zhang, J 2012, Proanthocyanidins inhibit seed germination by maintaining a high level of abscisic acid in *Arabidopsis thaliana*, *Journal of Integrative Plant Biology*, vol. 54, pp. 663-673.

- Karssen, CM, Brinkhorst-van der Swan, DLC, Breekland, AE & Koornneef, M 1983, Introduction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.), *Heynh, Planta*, vol. 157, no. 2, pp. 158-165.
- Planes, MD, Niñoles, R, Rubio, L, Bissoli, G, Bueso, E, García- Sánchez, MJ, Alejandro, S, Gonzalez-Guzmán, M, Hedrich, R, Rodgrigues, PL, Fernández, JA & Serrano, R 2014, A mechanism of growth inhibition by abscisic acid in germinating seeds of *Arabidopsis thaliana* based on inhibition of plasma membrane H<sup>+</sup>-ATPase and decreased cytosolic pH, K<sup>+</sup>, and anions, *Journal of Experimental Botany*, vol. 66, pp. 813-825.
- Rahsotte, A, Jenks, M, Feldmann, K 2001, Cuticular waxes on *eceriferum* mutants of *Arabidopsis thaliana*, *Phytochemistry*, vol. 57, no. 1, pp. 115-123.
- Shrestham P, Callahan, DL, Singh, SP, Petrie, JR & Zhou, X 2016, Reduced triacylglycerol mobilization during seed germination and early seedling growth in *Arabidopsis* containing nutritionally important polyunsaturated fatty acids, *Frontiers in Plant Science*, vol. 7, pp. 2371.
- Zhao, Y 2012, A chemical genetics method to uncover small molecules for dissecting the mechanism of ABA responses in *Arabidopsis* seed germination, *Methods in Molecular Biology*, vol. 876, pp. 107-116.
- Zheng, H, Owen, R & Kunst, L 2005, Disruptions of the *Arabidopsis* enoyl-coa reductase gene reveal an essential role for very-long-chain fatty acid synthesis in cell expansion during plant morphogenesis, *The Plant Cell*, vol. 17, no. 5, pp. 1467-1481.