

Effect of *Cer10* mutation and light intensity on *Arabidopsis thaliana* water-uptake rates

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Abstract

The *Arabidopsis thaliana* wild-type plant produces cuticular wax on its exterior surface to serve many functions, including the regulation of water loss. Presence of the *cer10* mutation results in a decreased amount of cuticular wax production. In order to determine both the individual and interdependent effects of the *cer10* mutation and light intensity on the water-uptake rate of *A. thaliana*, we conducted water-uptake experiments on four treatments of *A. thaliana* plants with six replicates each: wild-type light, mutant light, wild-type dark, and mutant dark. Plants were kept in a 20°C incubator for 72 hours prior to the experiment in their respective light conditions. Water-uptake rate was calculated using the time required for *A. thaliana* to uptake the methylene blue indicator to its bottom-most leaves, the uptake distance. The mean water uptake rates for wild-type light, mutant light, wild-type dark, and mutant dark plants were found to be 42 µm/s, 30 µm/s, 26 µm/s, and 62 µm/s, respectively. No statistically significant differences were observed between light and dark treatments or wild-type and mutant plants; however, the differing effect of light and dark treatments on wild-type and mutant plants was significant (p -value = 0.026). The latter result was not as predicted, in that increased light intensity led to decreased, instead of increased, water-uptake rates of mutant plants, and increased water-uptake rates of wild-type plants. This may have been due to unaccounted-for physiological factors.

Introduction

Arabidopsis thaliana is a small flowering plant from the Brassicaceae family (Washington University 1993). The *A. thaliana* plant is the most widely studied plant in biology, found naturally in most places throughout Eurasia, and can be grown in a variety of environments (Courteau 2012, Cao *et al.* 2011). Part of the reason *A. thaliana* can survive in most environments is its ability to form cuticular waxes, which helps prevent non-stomatal water loss (Le Provost *et al.* 2013). In this experiment, we tested *cer10* *A. thaliana* mutants, where the gene coding for enoyl-CoA reductase (ECR), used for long chain fatty acid synthesis, has been inactivated by gene knockout (Zheng *et al.* 2005). In *A. thaliana*, cuticular waxes are formed by synthesizing C24 to C32 fatty acids, which are then modified to make the primary alcohols used to produce cuticular wax (Lai *et al.* 2007). Plants with the *cer10* mutation were found to have a partial depletion of the C26 and C28 alcohols used for cuticular wax production, leading to less cuticular wax than wild-type plants (Lai *et al.* 2007).

Our primary objective in this research was to determine if there was a significant difference in the water-uptake rate of the *A. thaliana* wild type as compared to the *cer10* mutant. Our second objective was to investigate if light intensity has an effect on *A. thaliana* water uptake. In conjunction, these two objectives formed our third objective, which was to determine the difference by which light intensity affects the *cer10* and wild-type *A. thaliana* plants. Thus, this study allowed us to improve our understanding of the effects of the *cer10* mutation and light intensity, both in isolation and in conjunction with one another, on the water-uptake rate of *A. thaliana*.

Our null hypothesis H_{o1} was that the *cer10* mutation does not have an effect on *A. thaliana* water-uptake rate. Our alternate H_{a1} hypothesis was that the *cer10* mutation does have an effect on *A. thaliana* water-uptake rate. Our null hypothesis H_{o2} was that light intensity does not have an effect on *A. thaliana* water-uptake rate. Our alternate hypothesis H_{a2} was that light intensity does have an effect on *A. thaliana* water-uptake rate. Our null hypothesis H_{o3} was that the effect of light intensity on the water-uptake rate is the same in *A. thaliana* wild type and *cer10* mutant. Our alternate hypothesis H_{a3} was that the effect of light intensity on the water-uptake rate is not the same in the *A. thaliana* wild-type and *cer10* mutant plants.

We predicted that the *cer10* mutant plants would have a higher water-uptake rate as they accumulate less cuticular wax compared to the wild type and will therefore lose water to the environment more readily (Zheng *et al.* 2005). This is because cuticular wax helps prevent non-stomatal water loss; plants with more wax will therefore have less water loss and require less water uptake (Le Provost *et al.* 2013). We also predicted that light intensity would increase water-uptake rate in *A. thaliana* plants. Plant water loss is greatly reduced when stomata are closed (Boyer *et al.* 1997), which occurs when *A. thaliana* plants are placed in a dark environment for an extended period of time (Boyer *et al.* 1997, Jeon *et al.* 2008).

Thus, *A. thaliana* will lose more water in an environment with greater light intensity and will have increased water uptake to compensate.

Lastly, we predicted that there will be a difference in the way water-uptake rate is affected by light intensity in *A. thaliana* wild-type plants compared to *cer10* mutant plants. Specifically, we predicted that increased light intensity will result in a greater increase in the water-uptake rate of the mutant as compared to the wild type. This is because the mutant accumulates less cuticle wax, resulting in greater non-stomatal water loss (Le Provost *et al.* 2013). Boyer *et al.* (1997) showed that water loss from the cuticle ranges from 1.7% to 28.6% of the water loss that occurs in the stomata. That ratio increases significantly as the stomata close, causing water transfer to become more cuticle-dependent. Figure 1 summarizes the predictions that light should cause the light-treatment plants' stomata to open, causing both stomatal and cuticular water loss, whereas the dark-treatment plants should only experience cuticular water loss. Due to the open stomata, all light treatment plants were predicted to have water-uptake rates exceeding those of dark-treatment plants. Furthermore, mutant plants should have water-uptake rates exceeding those of the wild-type plants in the same light conditions, due to lower wax accumulation and greater subsequent cuticular water loss (Boyer *et al.* 1997).

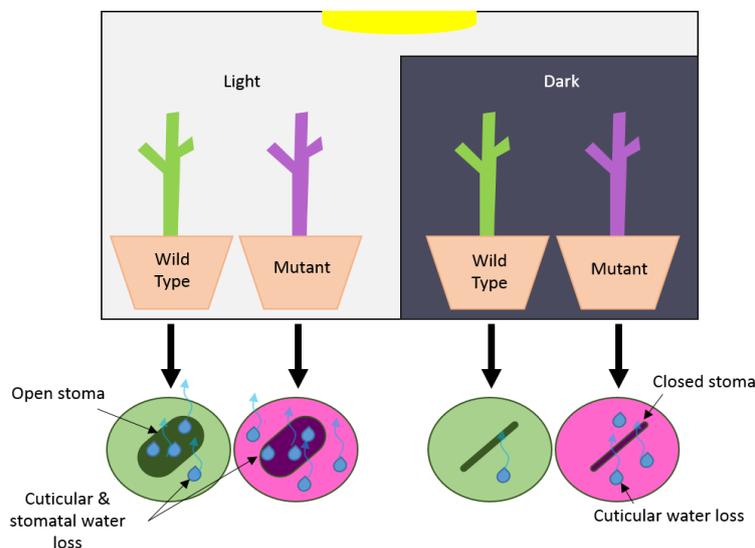


Figure 1. Effect of light intensity on wild-type and mutant *A. thaliana* plants.

In addition to cuticular wax accumulation, the *cer10* gene is implicated in hindering the development of the leaf epidermis, where the stomata are found (Zheng *et al.* 2005). , It may disrupt the functionality of ROP2, a Rho GTPase (ROP) found in the guard cells of *A. thaliana* (Zheng *et al.* 2005). ROP2 is involved in epidermal cell growth, and is thus vulnerable to the effects of *cer10* inactivation; however, it primarily functions to regulate stomatal movements by preventing excess stomatal opening in bright light and inducing stomatal closure in the dark (Jeon *et al.* 2008). Therefore, loss of ROP2 functionality may result in open stomata in the mutant dark-treatment plants, leading to increased water loss and increased water uptake to compensate. This effect is depicted in Figure 2.

It was also noted that *cer10* mutants may have reduced capacity for auxin endocytosis, a major plant hormone (Zheng *et al.* 2005). Smalle and Van Der Straeten (2003) performed studies on auxin-deficient plants and found that they had slightly larger leaf surface areas, on the order of several percentage points. This may cause our mutant plants to have slightly larger surface areas over which water loss may occur. Auxin is also involved in the growth and differentiation of xylem, the vascular tissue responsible for water transportation in plants (Fabregas *et al.* 2015). Thus, auxin deficiency may impede xylem development in our mutant plants, and therefore reduce their physiological capacity for water uptake (Figure 3).

As these side effects of *cer10* inactivation are antagonistic to one another, it is unclear how they will cumulatively manifest in our results. However, we expect that the combination of reduced cuticular wax, ROP2 functionality loss, and auxin deficiency-induced leaf area increase will result in the *cer10* mutant having a greater water uptake rate in spite of the lack of xylem development due to auxin deficiency.

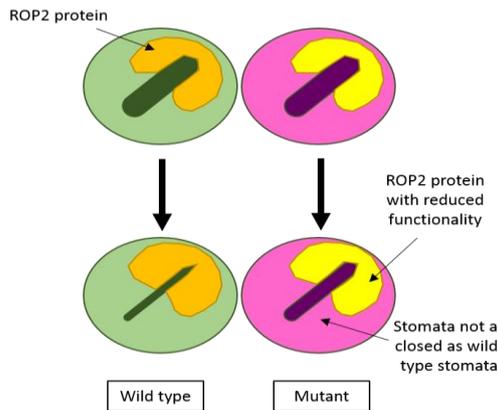


Figure 2. Effects of *cer10*-induced ROP2 functionality loss.

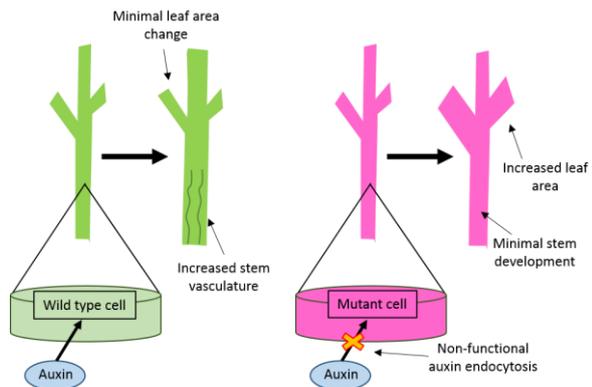


Figure 3. Effects of *cer10*-induced auxin endocytosis functionality loss.

Methods

Preparation

We first prepared two pots of soaked soil for planting *A. thaliana* seeds. After soaking for 24 hours, we verified soil moisture saturation using a soil moisture meter. We then planted equal numbers of wild-type and mutant seeds in two separate, labelled pots. We allowed the plants to grow in the pots for five weeks and then transferred them into four new pots in order to separate them for the various treatments. The new pots were pre-soaked in the same manner as the original pots. Each pot contained six seedlings, with two of the pots containing only wild-type plants and two of the pots containing only mutant plants. One pot of each genotype was kept in the dark, and the other in the light, such that our four pots were categorized as wild-type light, mutant light, wild-type dark, and mutant dark.

The pots were then placed in a 20°C incubator; plants subjected to the dark treatment were covered by a cardboard box. This temperature was closest to the optimal *A. thaliana* growth temperature of 23-25°C (Salinas 2005) (Figure 4). The light intensity was 4250 lux in the incubator and 3 lux under the cardboard box in the incubator. Plants remained in the incubator in light and dark conditions for 72 hours to ensure total stomatal closure (Seo *et al.* 2011).

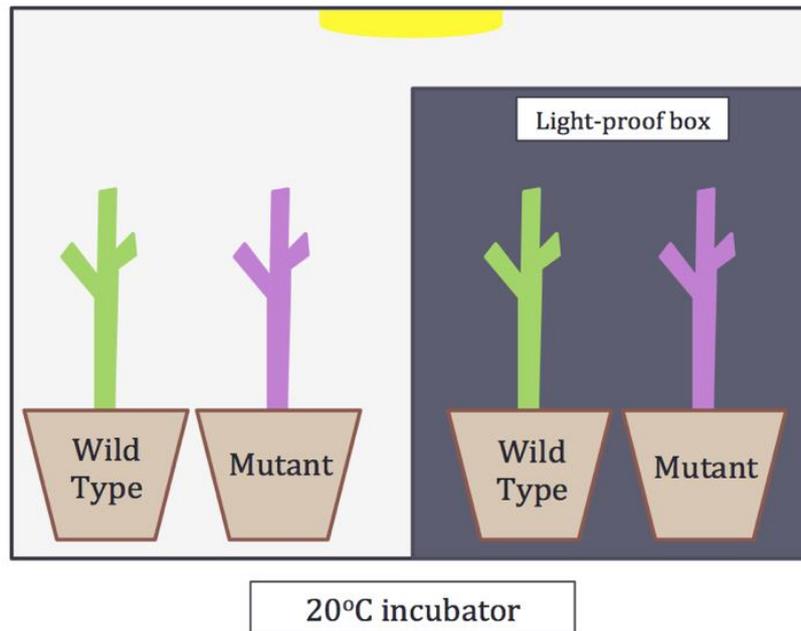


Figure 4. Organization of *A. thaliana* mutant and wild-type plant treatments in 20°C incubator.

After 72 hours, we took the plants out to perform the experiment. The light intensity was 2500 lux in the lab and 3 lux under the cardboard box in the lab. During the experiment, we took care to ensure that dark-treatment plants were promptly replaced underneath the cardboard box after extracting each sample.

Experiment

We extracted sample plants from the soil individually as needed. Each operator only tested one plant at a time, and we rotated the plant treatments each operator tested in order to minimize operator variability. We lightly brushed off the plant such that the stem was clearly visible and free of dirt, cut the roots off the plant and laid the sample flat on a Petri dish. We then pipetted 10 μL of methylene blue onto the Petri dish such that the cut tip of the plant stem was just immersed in the dye, and simultaneously started the timer. We observed the dye uptake under the dissecting microscope (Figure 5); this was the only time when lamps were turned on. Once the dye reached the bottom-most row of leaves, we stopped the timer and recorded the time for each plant. We then measured the distance between where the stem was dipped with dye to the bottom-most row of leaves. The uptake time and stem measurements were recorded for all six replicates of the wild-type light, wild-type dark,

mutant light and mutant dark treatments. After all replicates were completed, we prepared cross-sections of wild-type and mutant plant stems and used a Dinoscope to take images of the cross sections and intact stems.

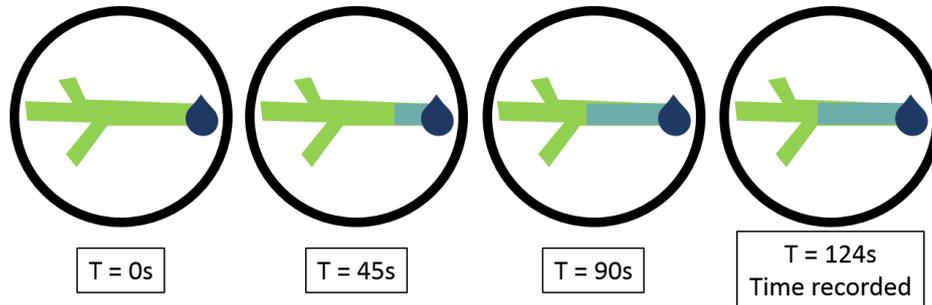


Figure 5. Representation of *A. thaliana* plant stem taking up methylene blue as viewed under the dissecting microscope.

Analysis

We calculated the uptake rate in micrometres per second. From this, we found the average, standard deviation, and 95% confidence intervals for the uptake rates of each treatment. We subsequently compared the treatment results via a two-way analysis of variation (ANOVA).

Results

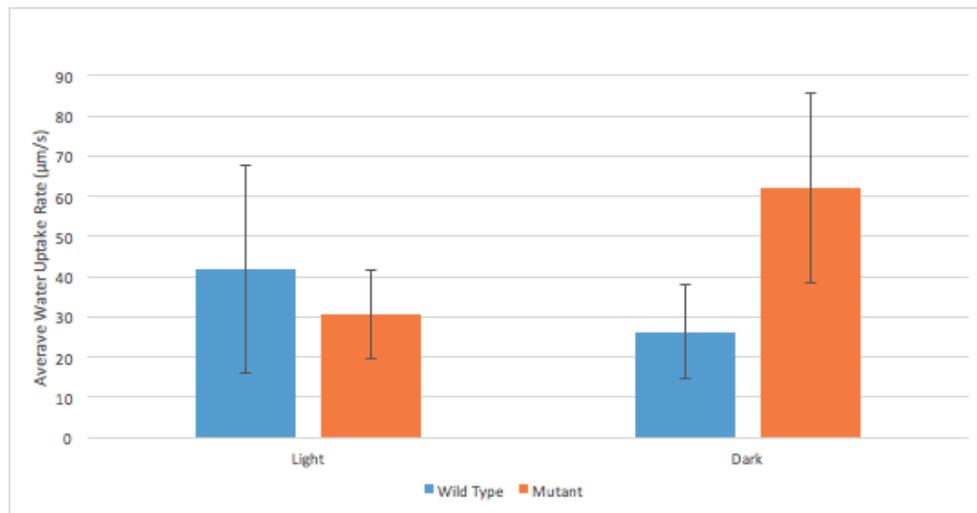


Figure 6. Average water uptake rates ($\mu\text{m/s}$) of wild-type and mutant *A. thaliana* plants in light (4250 lux) and dark (3 lux) conditions. Error bars represent 95% confidence intervals, $n=6$, $p=0.026$.

Figure 6 shows the mean water-uptake rates of wild-type and mutant *A. thaliana* plants in light and dark conditions. In light conditions, the wild-type plant has a mean rate of 42 $\mu\text{m/s}$ whereas the mutant has a lower rate of 30 $\mu\text{m/s}$. In dark conditions, the mutant plant

has a higher rate of water uptake of 62 $\mu\text{m/s}$ compared to the lower 26 $\mu\text{m/s}$ rate of the wild type. The p -value obtained from the two-way ANOVA statistical test for the effect of the *cer10* mutation on *A. thaliana* water-uptake rate was 0.22. The p -value obtained from the two-way ANOVA statistical test for the effect of light intensity on *A. thaliana* water uptake rate was 0.42. The p -value obtained from the two-way ANOVA statistical test for the interaction of the light and dark treatments with the mutant and wild-type treatments was 0.026. The 95% confidence intervals of the wild-type light, mutant light, and wild-type dark treatments overlap. The 95% confidence intervals for the mutant dark treatment do not overlap with the 95% confidence intervals of the wild-type dark treatment, but do overlap with the wild-type light and mutant light treatments.

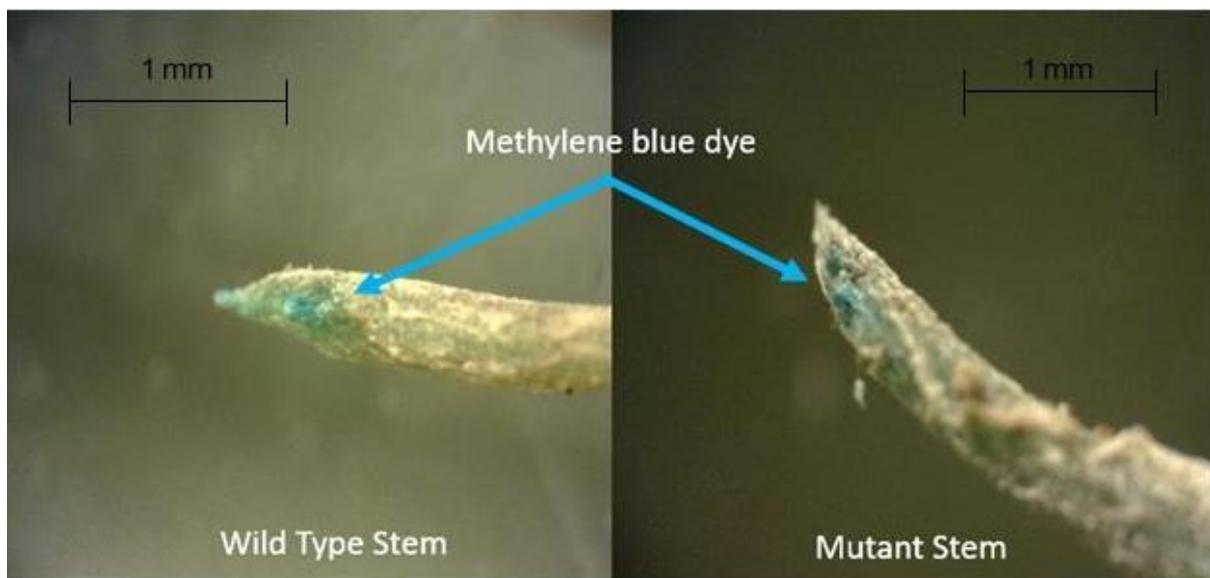


Figure 7. Interior of dyed and cut wild-type and mutant *A. thaliana* plant stems as viewed at 2x magnification on the dissecting microscope with DinoXcope.

Figure 7 shows a view of the interior of wild-type and mutant *A. thaliana* stems after the water uptake experiment was performed. The methylene blue can be seen as a distinctly teal-blue colour bound to the central tissue of the plant stems. Although not as pronounced in the Dinoscope photos, the wild-type plant stems tended to be thicker and more vascularized. Figure 8 shows dark treatment plants with their leaves upwards after 72 hours' exposure to low light conditions (3 lux).

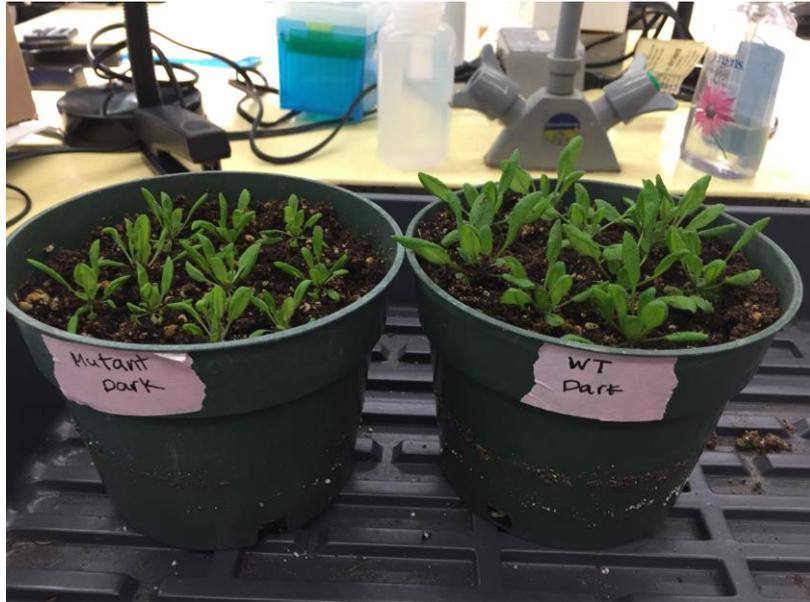


Figure 8. Wild-type and mutant *A. thaliana* plants exhibiting upward leaf orientation after 72 hours of exposure to low light conditions (3 lux).

Discussion

Based on our statistical analysis, we fail to reject H_{01} . The p -value of the two-way ANOVA test for the effect of the *cer10* mutation on *A. thaliana* water-uptake rate was 0.22, which is greater than 0.05. We originally predicted that the mutant plants would have a higher water-uptake rate than the wild-type plants. Although this predicted trend was observed in the dark treatments, the light treatments showed the opposite. The wild-type light plants had a higher average water uptake rate than the mutant light plants. We also failed to reject H_{02} . The p -value of the two-way ANOVA test for the effect of light intensity on *A. thaliana* water-uptake rate was 0.42, which is greater than 0.05. We initially predicted that *A. thaliana* would have a higher water uptake rate with the light treatments than the dark treatments; while this trend was observed for the wild-type plants, the opposite was observed for the mutant plants.

There are a number of factors, both physiological and experimental, that may have contributed to our results. The physiological factors are direct and indirect effects of *cer10* inactivation. The fact that the mutant dark water-uptake rate was the highest of all of the

treatments may be explained by a number of factors. The reduced amount of cuticular wax on the mutant plant due to the *cer10* mutation (Zheng *et al.* 2005), which functions to prevent the evaporation and loss of water from plants (Jenks *et al.* 2002), supports this result. Studies have shown that a greater amount of wax will reduce the water uptake rates of the plant (Ni *et al.* 2015). Therefore, the reduced amounts of cuticular wax on the mutant plants may have resulted in increased water loss, leading to an increased water uptake rate in response to dehydration. The wild-type plant did not experience as much water loss and consequently did not need to replenish its water content to the same extent and therefore had a slower water-uptake rate.

The physiology of Rho GTPases (ROP) in the guard cells of *A. thaliana* could have also contributed to this result. One such protein, ROP2, is a regulator of stomatal movements in response to light stimulus and is responsible for limiting stomatal opening in the presence of excess light, and inducing stomatal closure in the dark (Jeon *et al.* 2008). As the ROP2 protein is also involved in the growth of developing epidermal cells (Jeon *et al.* 2008), where the *cer10* mutation has been observed to stunt growth (Zheng *et al.* 2005), the mutant plants in our samples may have had non-functional ROP2 proteins. Therefore the mutant plants may have been unable to adequately close their stomata in the dark, which would have contributed to increased water loss, compared to the wild type.

Auxin deficiency may be another contributing factor to the observed results. Zheng *et al.* (2005) reported that *cer10* mutants may have reduced capacity for auxin endocytosis. Smalle and Van Der Straeten (2003) showed that auxin-insensitive plants may have slightly larger leaf surface areas as compared to wild-type plants due to auxin's role in bioallocation during growth. The difference may only be several percentage points, and may not be readily visible (Smalle and Van Der Straeten 2003). Due to the reduced auxin endocytosis capacity, it is possible that the mutant plants had slightly larger leaf surface areas and thus a greater

surface area over which to lose water to the environment. Auxin, in conjunction with ethylene, another plant hormone, is also responsible for the observed hyponasty phenomenon, where the leaves of all of the dark treatment plants were pointed upwards in a physiological attempt to receive more light (Smalle and Van Der Straeten 2003). The fact that hyponasty was observed potentially indicates that there was some auxin activity in the mutant plants (Smalle and Van Der Straeten 2003). While this may appear to contradict the previous explanation, the same study also indicated that some auxin-insensitive plants still exhibit hyponasty, and thus auxin deficiency-induced leaf surface area increases in our *cer10* mutants remains a possibility.

The experimental conditions may also partially explain our observed results. We were only able to use six replicates per treatment. If the number of replicates had been higher, a statistically significant difference may have been found between treatments. Also, the plants were kept in a shared incubator that contained uncovered trays of water, which may have increased the humidity for light treatment plants, whereas the dark treatment plants became more dehydrated within the confines of the box. This further explains the increased water-uptake rate of the mutant plant in the dark treatment.

The observed higher wild-type light water-uptake rate as compared to the mutant light water uptake rate is at least partially supported by literature and experimental error. In addition to leaf surface area regulation, auxin plays an important role in promoting the growth and differentiation of xylem, the vascular tissue responsible for the transportation of water in plants (Fabregas *et al.* 2015). Given the possibility that the *cer10* mutants may be auxin-deficient due to reduced endocytic capacity (Zheng *et al.* 2005), they may have less developed xylem tissue and thus reduced capacity for water uptake. This is consistent with our observations that wild-type plant stems were noticeably thicker and more vascularized than mutant stems.

The same factors discussed above which prevented us from rejecting H_{01} and H_{02} allow us to reject H_{03} and lend support for H_{a3} . These hypotheses respectively stated that the effect of light intensity on the water-uptake rate is not the same in *A. thaliana* wild-type and mutant plants. We were able to reject H_{03} as the p -value of the two-way ANOVA test for the effect of light intensity on mutant versus wild-type *A. thaliana* plants was 0.026, which is less than 0.05. However, our results contradicted our prediction as it was observed that increased light intensity led to water uptake rate increases with wild-type plants, but decreases with mutant plants.

The aforementioned physiological and experimental factors lend support to this result. The humidity differences between light and dark treatment plants due to the box placement may have hydrated the light treatment plants, but dehydrated the dark treatment plants. For the light treatment results, this would lead to an emphasis on physiological capacity for water uptake, as both groups of plants would be equally well-hydrated due to the greater ambient humidity. This can be seen in Figure 6, as wild-type light plants had a higher mean water-uptake rate than did mutant light plants, which, due to their auxin deficiency, may not have had as highly developed xylem. Conversely, the dark treatment results, due to the finite amount of water vapour, would be consistent for plants that were less able to control for water loss. In Figure 6, this is again the case, as the decreased wax accumulation, non-functional ROP2, and auxin deficiency-induced leaf area increase would have collectively led to greater dehydration in the mutant dark plants than in the wild-type dark plants.

With regards to the wide variation in our results, Pigliucci and Kolodynska (2002) found that there were few differences between *A. thaliana* grown in low and medium light intensity. Pigliucci and Kolodynska (2002) suggested that *A. thaliana* are able to adapt to the different light intensities. Therefore, this adaptability may have contributed to the variability observed in our results.

Experimental Error

Aspects that contributed to uncertainties may include the fact that some of the *A. thaliana* seeds used in this trial were planted over several days, due to the depletion of the *A. thaliana* plants that were planted on the same day during an unsuccessful trial. The slight age differences, as well as the increased competition induced by the plants' close-proximity during germination, may have induced an increased amount of developmental variability. The difference in maturity would affect the results directly, by having some plants with greater or lower physiological capacity for water uptake, and indirectly, as plants with greater vascularization would be more difficult to measure. Differences in age may also have affected leaf size, stomata counts, stem maturity, and cuticular wax load, which may all affect water uptake rates in *A. thaliana* (Kosma *et al.* 2009).

While we considered stomatal opening during the experiment as a potential source of error, we do not think this is likely to be a major contributor. Stomatal opening requires approximately 48 minutes to reach the half-maximum diameter (Jeon *et al.* 2008), and extra care was taken to ensure no dark treatment plants were exposed to light for longer than 3-5 minutes before being reintroduced to the dark environment for an equal period of time or longer.

Some error may also have been caused by operator variability. Visual determination of the dye uptake endpoint varied from operator to operator as some found it easier than others to monitor the progress of the dye. In addition, the visibly increased vascularization of the wild-type plant stems made it more difficult to visually ascertain the progress of the dye, which accounts for some of the high variability in the wild-type light plant results. We minimized the effects of these variations by rotating operators such that each operator tested each treatment at least once and never more than twice.

Conclusion

We were unable to reject H_{01} and H_{02} , i.e., the *cer10* mutation does not have an effect on the water-uptake rate of *A. thaliana*, and light intensity does not have an effect on the water uptake rate of *A. thaliana*. We were able to reject H_{03} and support H_{A3} , i.e., light intensity affects the water uptake rate of wild-type and mutant *A. thaliana* plants differently. Although this difference occurred in the opposite direction as expected, with the wild-type plants having an increased water-uptake rate in higher light conditions and the mutant plants with a lower rate, the literature does provide some support for the results obtained.

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