Chloroplastic protein IOJAP is important for cold acclimation in *Arabidopsis thaliana*

By

AMANDA CAREY

Advisor: Andreas Nebenführ
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INTRODUCTION

Temperature is one of the most important abiotic factors determining the productivity and topographical distribution of plants. There are numerous injuries that can occur in plant cells when exposed to low temperatures, including but not limited to solute leakage due to enhanced permeability of membranes (Seo et al. 2010), reduced ATP supply, chlorosis (Ruelland and Zachowski 2010), growth retardation, and even death (Theocharis et al. 2012). When exposed to low non-freezing temperatures, many plant species can trigger physiological and molecular changes to cope with the stress of the cold (Smallwood and Bowles 2002; Zhu et al. 2007), a process known as cold acclimation. There are several cellular mechanisms that are activated as plants become acclimated to low temperatures. The plasma membrane is sensitive to cold temperatures, and because it is the first site of cold-induced injury (Steponkus, 1984), changes to the cell membrane are the primary mode of action. A change in lipid composition of the membrane, mainly the increase in unsaturated fatty acids, allows for increased fluidity (Uemura and Steponkus 1999). In non-acclimated cells, low temperatures would cause the membrane to become rigid which allows the influx of calcium and disrupted ion balance. The increase in cellular calcium levels can trigger a cascade of low temperature responses (Sangwan et al. 2002), leading to the activation of cold responsive (COR) genes (Chinnusamy et al. 2007, 2010) and thus cold-acclimation (Theocharis et al. 2012).

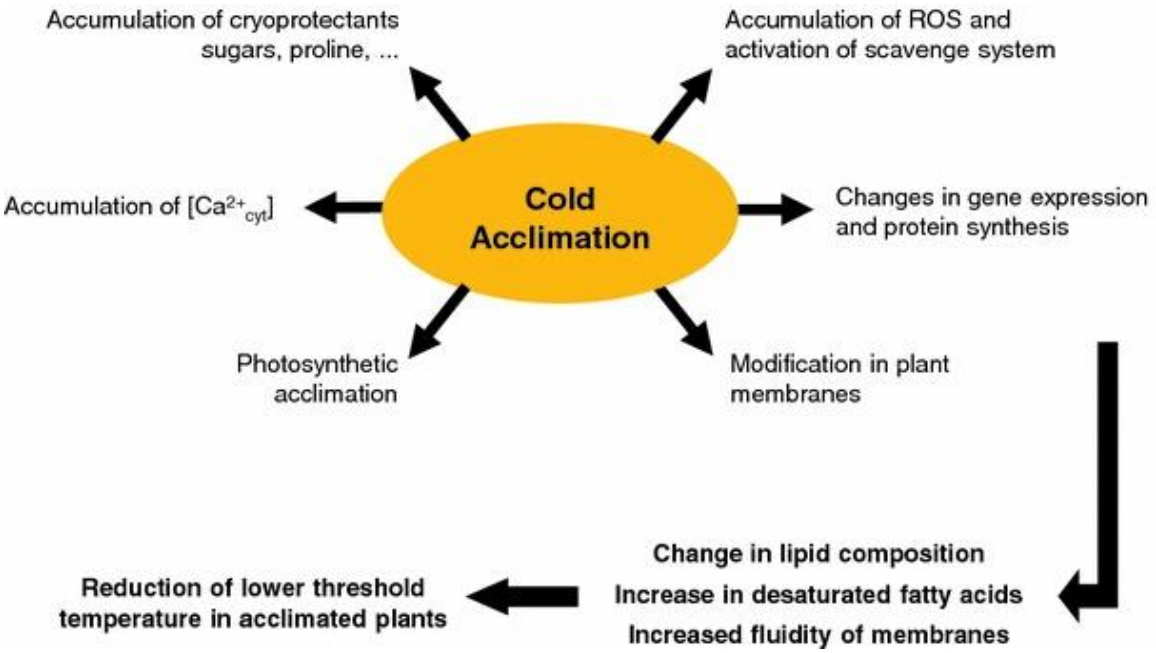


Figure 1: Various cellular processes triggered by plant reaction to cold temperatures (Theocharis et al. 2012).

Under conditions of great stress, such as cold, plants can experience impaired and abnormal cellular function, causing changes to the metabolism. The metabolism becomes slower, thus reducing the growth rate and as well as the chloroplast function. This is partly a result of reduced reaction rates and enzyme activity. One example of reduced metabolism is seen in *Arabidopsis thaliana*, where there is an inhibition of photosynthetic gas exchange in the leaves of cold-stressed plants (Savitch et al. 2001). However, if the plant is cold acclimated, it is possible for the cellular processes to continue until the plant's minimum temperature for survival is reached. At that point, the physiological, cellular, and molecular dysfunction becomes so severe that death or stasis is inevitable.

Arabidopsis thaliana, as mentioned previously, will experience a decrease in enzymatic activity and function when placed in the cold, but this plant also has the ability to cold-acclimate. Genes activated in the cold to start and aid in cold-acclimation can be regulated by multiple proteins. One cold acclimation pathway is controlled by the C-

repeat binding factors (CBFs) (Gilmour et al. 1998) which are induced by the inducer of CBF expression 1 or (ICE1) (Chinnusamy et al. 2003). These CBFs (1, 2 and 3) are regulatory proteins that control the expression of the CBF regulon, which is the range of COR genes that contribute to cold acclimation. New results, however, provide evidence for a more complex path to cold acclimation than the CBF regulatory pathway alone. Park et al. (2015) found that about 25% of the genes that make up the CBF2 regulon belong to at least one other regulon, which is controlled by multiple first wave transcription factors. There is much to be discovered about the highly complex process of cold acclimation.

A chloroplastic protein known as IOJAP is found in *Arabidopsis*. A homolog of this gene is found in *Zea mays*, where the encoded protein is localized in plastids and associates with the 50S large ribosomal subunits (Martienssen 2001). There is 65% sequence similarity between the IOJAP homologs in *Z. mays* and *A. thaliana* (ClustalW). We assume that they both function in the chloroplast, and would therefore have similar roles. Mutation in this *iojap* gene in *Z. mays* leads to variegation in leaves, caused by defects in chloroplasts. Variegation does not occur in mutant *ijT1* in Arabidopsis, which is a mutation by T-DNA insertion in the first intron. Some discoloration is seen in this mutant, along with aberrant morphology in young leaves. Interestingly, the cold pathway does not regulate *IOJAP* transcription levels. It was previously shown that plants contain double mutant *iojap* and myosin *xik-3* were cold sensitive. However it was soon found that cold sensitivity was most likely caused by *iojap* instead of *xik-3*. The exact causes of this are still unknown.

In order to continue the life cycle, plants need to produce seeds. The inflorescences of *Arabidopsis thaliana* are crucial to the seed development process. Branches growing out from the main inflorescence stem have siliques, which hold the seeds needed for reproduction. This seed development process can be separated into three stages: early embryogenesis, maturation, and late maturation (Baud et al. 2001). The defected chloroplasts in *iojap* mutant *Z. mays* could affect seed vitality, since one important role of chloroplast is harvesting energy. In this experiment, we viewed seed mass as an indicator of seed health; we analyzed the seeds produced from wild type Col-0 and *ijT1 Arabidopsis* plants grown in normal temperatures (22℃) and low but not freezing temperatures (12℃ and 4℃). This research will also examine phenotypes in order to further describe the *IOJAP* mutant in Arabidopsis and the way cold affects the plant.

METHODS

Seed Preparation. Wild type *iojap* (WT-Col-0) and mutant *iojap* (*ijT1)* seeds were surface sterilized with 30% bleach, 0.1% Triton X-100 solution for 10 minutes. After stratification at 4℃ for 48 hours, seeds were plated on phytagel plates (1% sucrose, 1/4xMS salts, 0.5% phytagel, pH 6 with KOH) then germinated on vertical plates at 22℃ on long day cycle (16 hours of light, 8 hours of darkness) for 48 hours.

Root Length Analysis. Following germination, plates were photographed individually then placed in designated temperature chambers, i.e. 4℃, 12℃, and 22℃. Photographs were taken every day at the same time, plus or minus an hour, then ImageJ

was used to measure the root lengths. Photos were taken until the root was no longer measurable. Growth rate and root length at each temperature was analyzed using multiple t-tests (one per row) and a Tukey test. The entire experiment was repeated twice.

Leaf Appearance. Seeds were plated on horizontal phytagel plates and moved to their designated temperatures (22°C, 12°C, and 4°C) after germination. Plates were monitored daily in order to determine when leaves were ready to be photographed using the stereoscope. Images were taken at cotyledon stage (when >2 mm), when the first pair of true leaves emerged (>2 mm) and when the second pair of true leaves emerged (>2 mm).

Inflorescence Height and Rosette Radius. After germinating for 5 days, seeds were transferred to soil (Fafard Growing Mix). The moisture level of the soil was wet but not saturated. Soil was transferred to small pots of a volume ~91 mL, which were numbered 1-9 for each temperature and placed in a plastic plant tray. A solution of 0.5 tsp of Miracle Grow and 3 liters of water was made, and then 1 liter of the solution was given to each tray. Next, another 1 liter of just water was given to each set of pots. The three plant trays were kept at 22°C for 48 hours, and then they were placed into the incubators of their designated temperatures. The water level in each tray was monitored every day, and once the tray became dry, the plants were given 1 liter of water the following day. Every other time the tray became dry the plants were given 0.5 tsp of Miracle Grow in 1 liter of water. Plants were monitored every day, and on the first day of inflorescence emergence pictures were taken from above and included a scale in order to measure the radius of the rosettes. The number of leaves was also recorded at the time of

inflorescence emergence. The rosette radii of both genotypes were measured using ImageJ and the averages were analyzed using an unpaired t-test with equal standard deviation. Once the inflorescences were at a height at which they began to lean they were loosely tied to bamboo sticks placed in the pots to keep them from becoming entangled with surrounding plants.

For plants grown at 22℃, 10 days after inflorescence emergence a second picture was taken from a tripod in order to get the side view of the growing inflorescence and measure its height. For plants grown at 12℃ the second pictures were taken 40 days after inflorescence emergence using the same method. Once the plants had grown to what was assumed to be full size, they were no longer given water and allowed to finish their life cycle.

Seed Analysis. Seeds were harvested from WT and *ijT1* plants grown at 22°C and 12℃ on long day cycle (16 hours of light, 8 hours of darkness) at the end of their life cycle. In order to measure relative seed size, seeds were sieved through mesh plates. The largest filter size was 300 micrometers, meaning the large seeds were greater than 300 micrometers. The medium size seeds were between 250 and 300 micrometers, and the small seeds were less than 250 micrometers. After the harvested seeds from each plant were separated, the mass of 100 seeds was taken from each of the small, medium and large categories. The average mass of 100 seeds from each size category from the 10 WT plants was calculated and compared to that of each size category from the 10 *ijT1* plants using a one-way Anova statistical test. Then, using the camera attached to a stereoscope, they were viewed under a magnification of 5 to look for qualitative differences. A picture of roughly ten seeds was taken of each size category for each of the 20 total plants.

RESULTS

Root Length Analysis. In order to confirm previous results showing a difference between the genotypes in the growth rate, the root growth was observed. When grown at 22°C, the root length of WT and *ijT1* were not different. The average of the final root length on day 11 was 76.9 mm for WT and 77.5 mm for ijT1. The growth rates followed the same pattern as well, with no significant difference between the two genotypes (**Figure 2**).

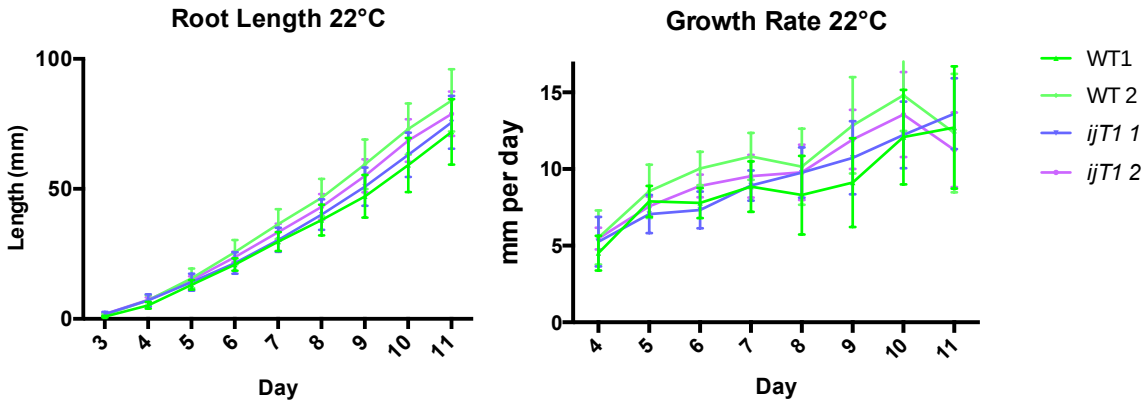


Figure 2: The root length in mm and growth rate in mm per day of *ijT1* and WT at 22°C. The green lines show WT for both replicates, while the purple lines show *ijT1*.

When the plants were grown in lower temperatures (12°C and 4°C), a significant difference between the genotypes in both growth rate and overall root length was seen. In the 12°C group, the root lengths of WT and *ijT1* plants were significantly different starting on day 9; the growth rates of WT and *ijT1* were significantly different from day 6 forward (**Figure 3**). The average final root length of *ijT1*, on day 20, was half of the final root length of WT (51.4% decrease in *ijT1*).

The growth rates for WT and *ijT1* incubated at 22°C and 12°C were also quite different. The graphs show that the slopes of the lines varied greatly. The growth rate of

WT at 12°C increased until around day 12 and at that point the growth rate started to decrease, whereas the growth rate of *ijT1* increased just briefly at the beginning and then decreased dramatically before leveling out around day 7. On the other hand, the slope of the lines representing growth rate for WT and *ijT1* at 22°C was continuously positive overall. The average growth rate from day 4 to day 12 for WT at 12°C was 2.37 mm/day and for *ijT1* it was 1.33 mm/day. The average growth rate from day 13 to day 20 for WT at 12°C was 2.13 mm/day while the average growth rate for *ijT1* during the same time was 1.0 mm/day.

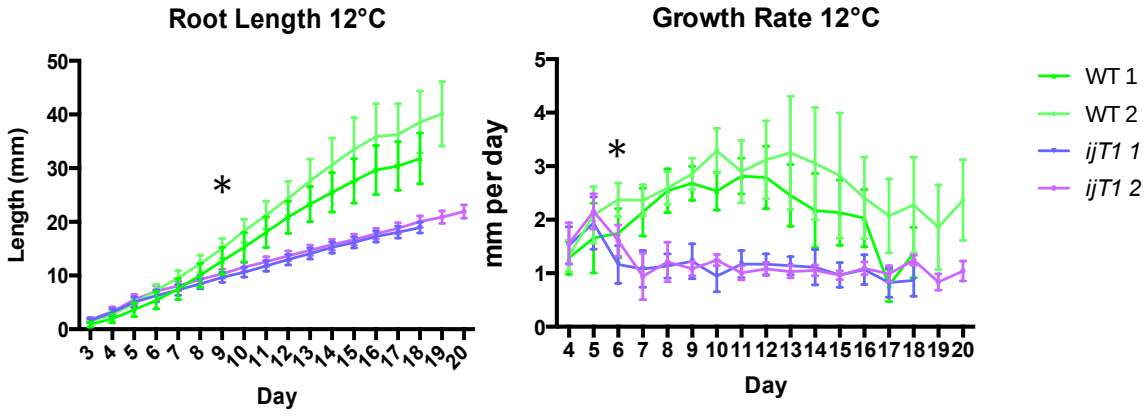


Figure 3: The root length in mm and growth rate in mm per day of *ijT1* and WT at 12°C. The green lines represent WT for both replicates, while the purple lines represent *ijT1*. The days where the genotypes started to be significantly different are labeled on graphs with *.

Seedling roots grew much more slowly at 4°C. The root lengths of WT and *ijT1* for the first replicate of 4°C (WT 1 and *ijT1* 1) were significantly different beginning with day 33, while the second replicate (WT 2 and *ijT1* 2) showed a significant difference in root length starting with day 30 (**Figure 4**). The growth rates of WT and *ijT1* for the first replicate were significantly different starting with day 18, while the second replicate shows a significant difference in growth rate beginning on day 30.

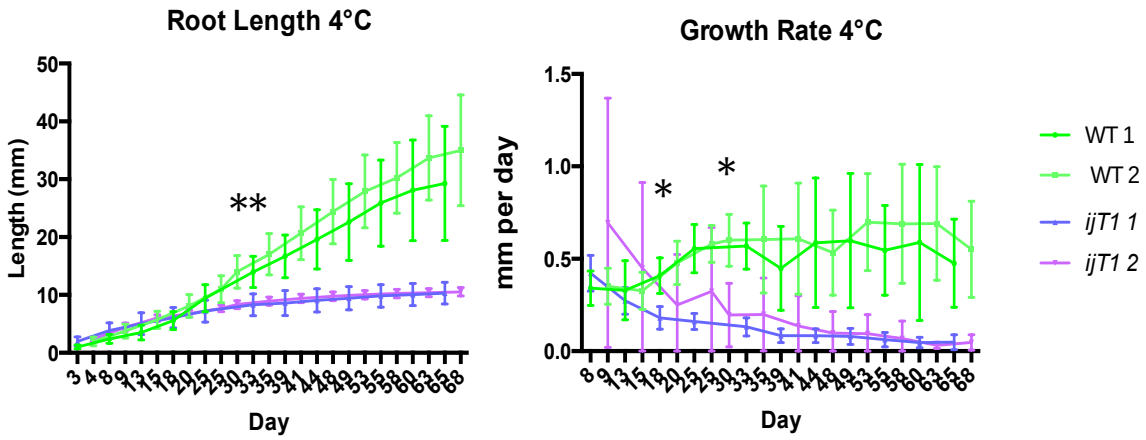


Figure 4: The root length in mm and growth rate in mm per day of *ijT1* and WT at 4°C. The green lines show WT for both replicates and the purple lines represent the *ijT1* replicates. The days where the genotypes were first significantly different are labeled on graphs with *.

Leaf Analysis. Because the ijT1 mutant Arabidopsis plants have shown irregular leaf morphology in the past when grown in low temperatures, the young leaves were observed to look for differences between those grown at 22°C and 12°C. In warmer temperatures, like 22°C, the phenotype of the *ijT1* leaves was normal, meaning they were green in color, and relatively flat. When the plants were grown in low temperatures 12°C and 4°C, the *ijT1* leaves were more yellow and not as flat; they were curled up, referred to as hyponastic, and smaller than those grown at 22°C. Hydathodes—the pointed extensions toward the base of the leaves seen in **Figure 6 b**—were present in leaves grown at 12°C but not 22°C.

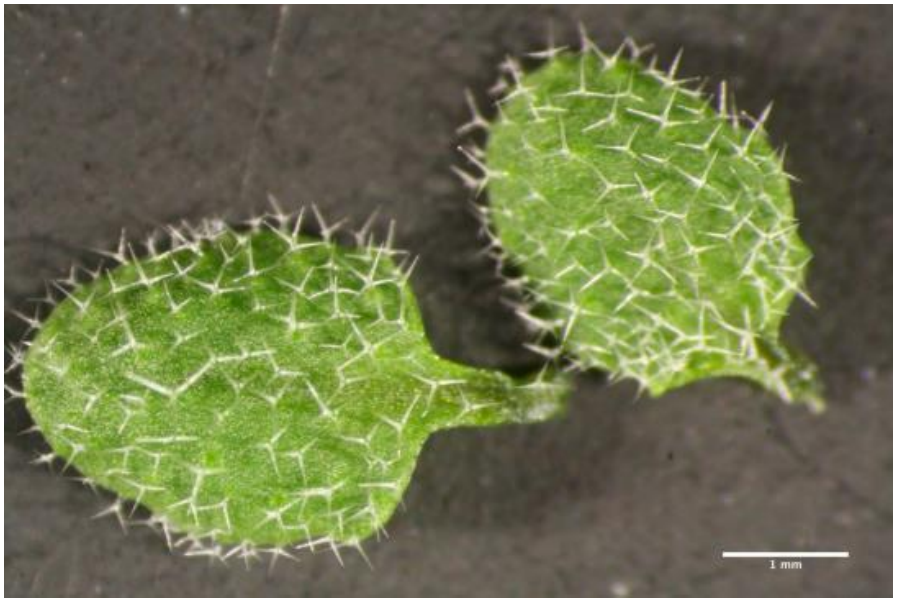


Figure 5: The first true leaves of *ijT1* at 10 days after germination grown at 22°C.

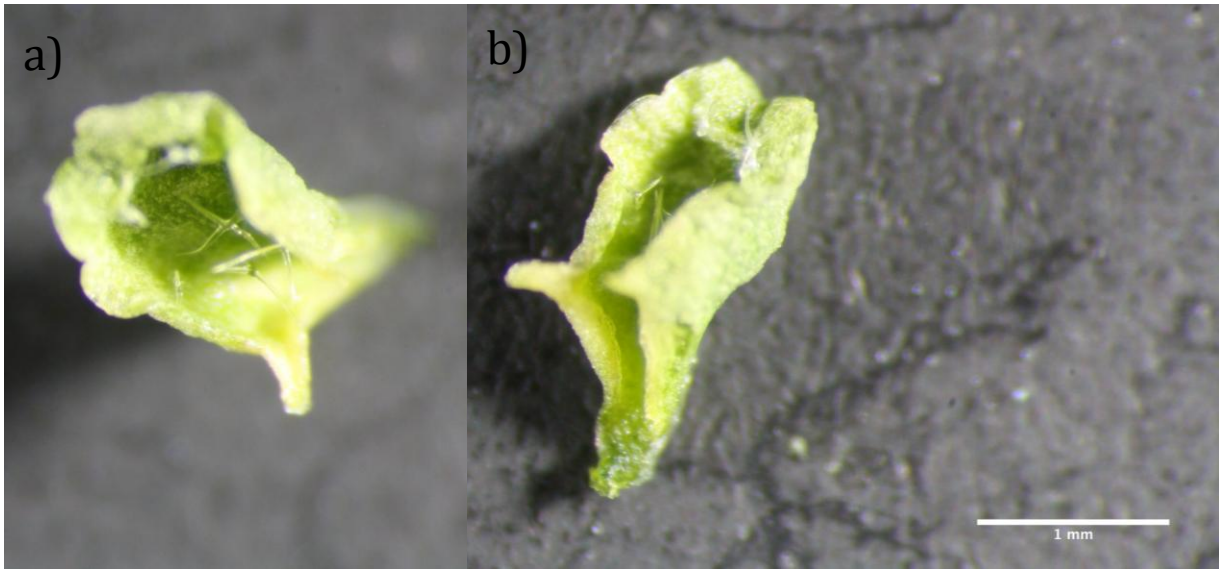


Figure 6. The first true leaves of *ijT1* at 19 days after germination grown at 12°C.

Rosette Radius Analysis. In order to see if the general size of mutant plants was affected in the cold, the rosette radius was measured. On the day of inflorescence emergence, there was not a significant difference between the number of leaves present in the WT and *ijT1* plants grown at both 22°C and 12°C. For the plants grown in 22°C, the

average leaf number for WT was 9.7 +/- 0.37, and that of *ijT1* was quite similar at 9.3 +/- 0.41 (**Figure 7 a.**). For plants grown in 12°C, the average for WT was 16 +/- 0.9 leaves, while the average for *ijT1* was 13.6 +/- 0.7 leaves (**Figure 7 b.**). The rosette radii, however, differed in both 22°C and 12°C; on average the genotypes were significantly different, with WT plants having a larger rosette radius than *ijT1* in 22°C ($p=0.0034$) and 12°C ($p<0.0001$). The average rosette radius for WT plants at 22°C was 26.4 +/- 1.2 mm while the average radius for *ijT1* plants was 19.8 +/- 1.5 mm (**Figure 8 a.)**. In plants grown at 12°C, the average rosette radius was 21 +/- 0.8 mm in WT and 15 +/- 0.7 mm in *ijT1* (**Figure 8 b.**).

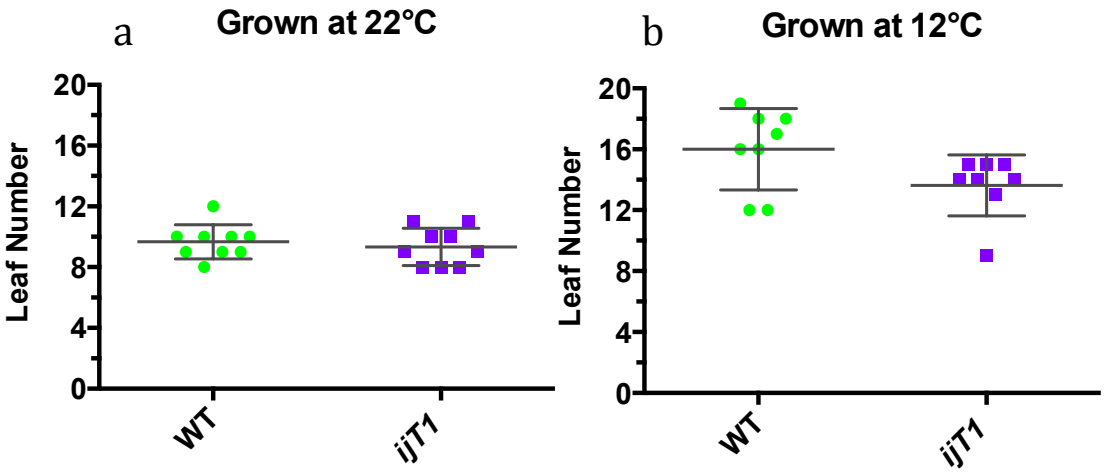


Figure 7: Number of leaves present (including cotyledons) on the day of inflorescence emergence for 22°C (a.) and 12°C (b.) grown plants. WT is shown in green, and *ijT1* is shown in purple. Error bars represent standard deviation.

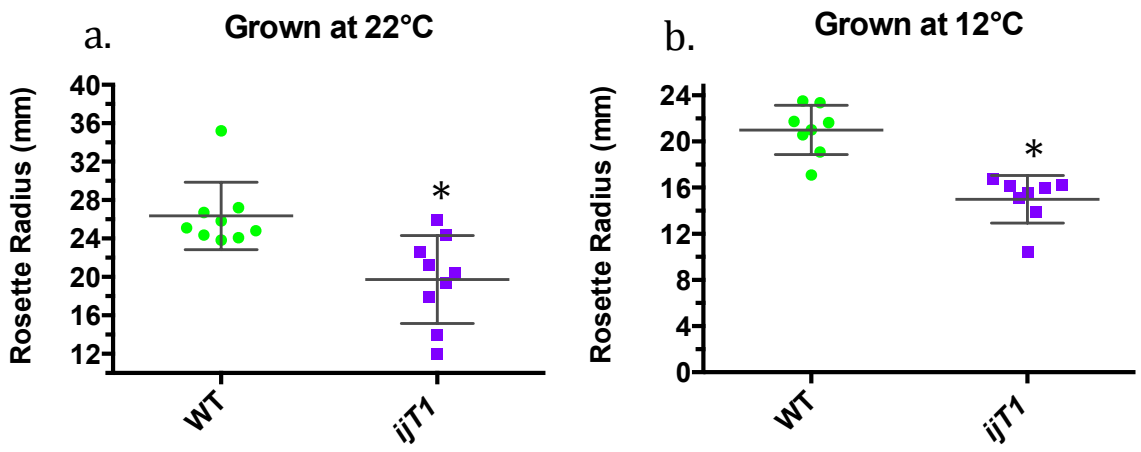


Figure 8: Distribution of rosette radii for WT and *ijT1* grown at 22°C (a.) and 12°C (b.). Green represents WT while purple represents the mutant, *ijT1*. Significant difference shown by *. (p=0.0034 and p<0.0001). Error bars represent standard deviation.

The inflorescence height was measured 10 days after bolting in 22°C and 40 days after bolting in 12°C. There was a significant difference between the two genotypes at both temperatures. The average inflorescence height for WT plants at 22°C was 20 +/- 0.6 cm while *ijT1* plants of 22°C had an average on only 15.6 +/- 1.3 cm (p=0.0059) (**Figure 9 a**.). WT plants at 12°C were also taller than *ijT1* plants in the same temperature; the average height for WT was 14.7 +/- 0.9 cm while the average inflorescence height in *ijT1* was 11.7 +/- 0.8 cm (**Figure 9 b.**). Like in 22°C, the difference was significant (p=0.0245).

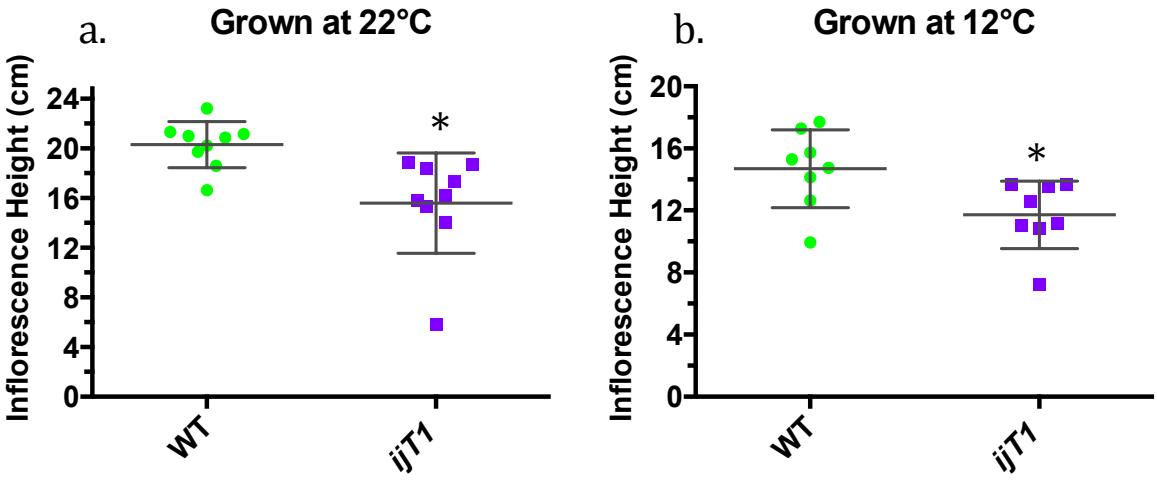


Figure 9: Distribution of inflorescence height of 22°C plants (a.) and 12°C plants (b.). WT plant heights are shown in green and *ijT1* plants are shown in purple. Significant difference shown by *. Error bars represent standard deviation.

Seed Analysis. To get a better idea of seed health and vitality, the mass of seeds was measured and compared between genotypes and temperatures. After harvesting seeds from 22°C and 12°C plants and separating them by relative size, the average mass of each of the three sizes was measured. In the seeds harvested from 22°C plants, WT small and *ijT1* small seeds were not significantly different in mass. The mass of 100 small WT seeds was 1.39 +/- 0.12 mg and that of small *ijT1* seeds was 1.44 +/- 0.16 mg. Likewise, there was no difference in the large WT seeds and large *ijT1* seeds. The mass for 100 large WT seeds was 2.71 +/- 0.31 mg while the mass for 100 large *ijT1* seeds was 2.42 +/- 0.21 mg. There was, however, a difference in the mass of the medium size for WT and *ijT1* seeds; the average mass for medium WT seeds was 2.22 +/- 0.13 mg and that of medium *ijT1* seeds was only 1.95 +/- 0.18 mg.

In the seeds harvested from 12°C plants, there was a difference in the mass of WT and *ijT1* seeds in the small and medium sizes, but not the large seed size. The average mass of 100 seeds from the small WT group was 1.2 +/- 0.15 mg and the average in the

small *ijT1* seed group was 1.46 +/- 0.22 mg. In the medium seeds, WT average mass was 2.01 +/- 0.12 mg while *ijT1* mass was 2.33 +/- 0.16 mg. The large seeds were not significantly different; WT average was 2.75 +/- 0.21 mg and *ijT1* average at 2.66 +/- 0.14 mg.

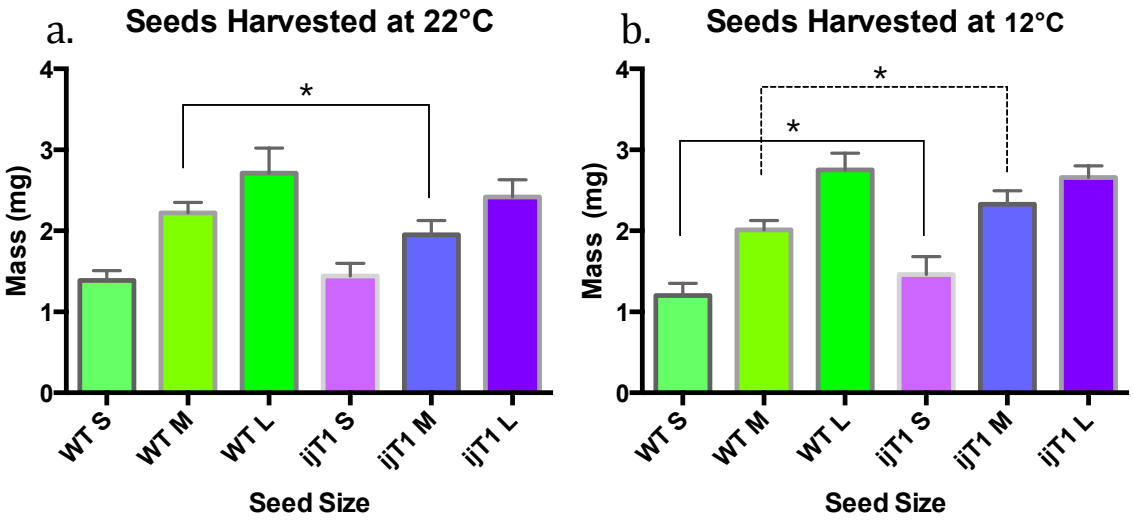


Figure 10: Average mass of 100 seeds collected from plants grown in 22°C (a.) and 12°C (b.) Three sizes of WT (small, medium, and large) are shown in green, and three sizes of *ijT1* are shown in purple. Significant difference shown by *. Error bars represent standard deviation. (n = 20 plants at each temperature).

Pictures were taken of seeds in order to compare the difference in appearance between small, medium and large seeds of both genotypes grown in 22°C and 12°C.

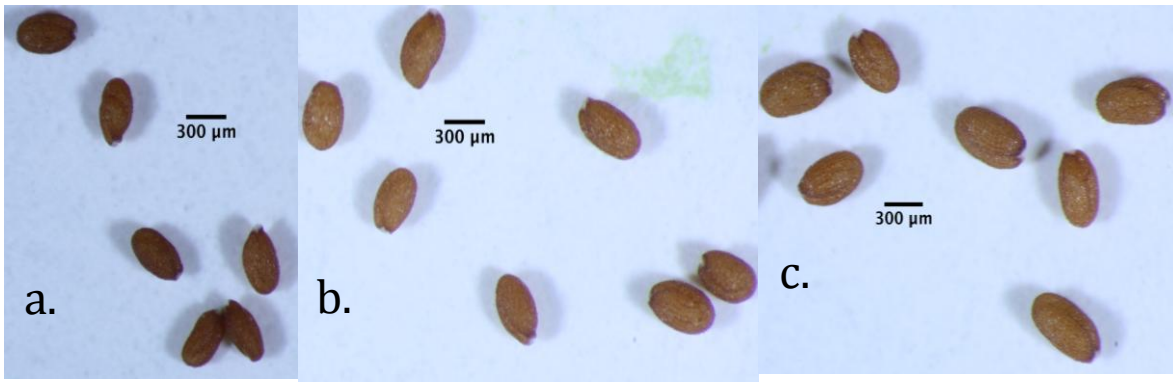


Figure 11: Seeds harvested from a WT plant grown in 22°C. a. is small, b. is medium, and c. is large.

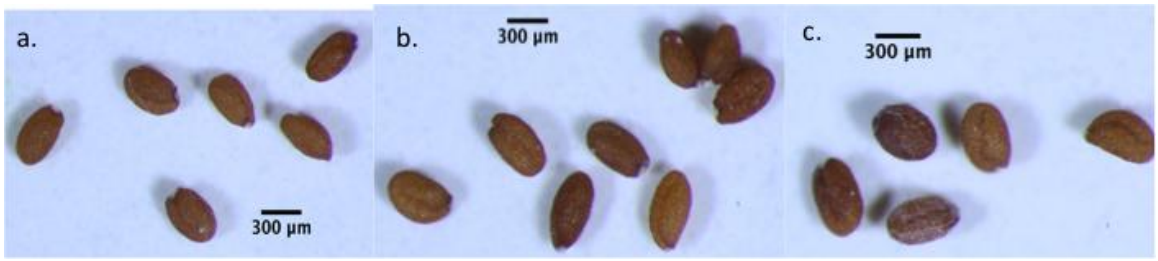


Figure 12: Seeds harvested from an *ijT1* plant grown in 22°C. a. is small seeds, b. is medium, and c. is large.

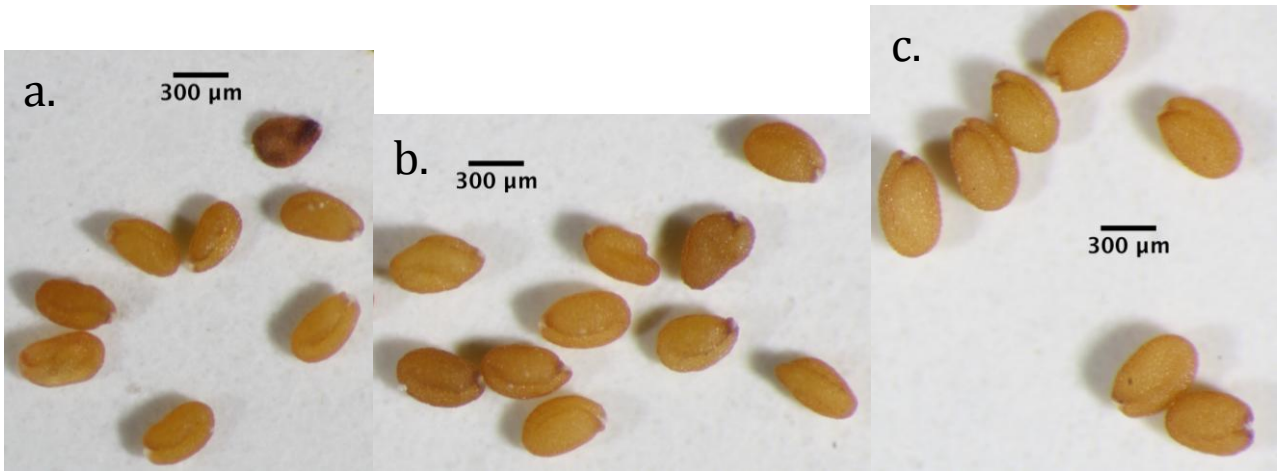


Figure 13: Seeds harvested from a WT plant grown at 12°C

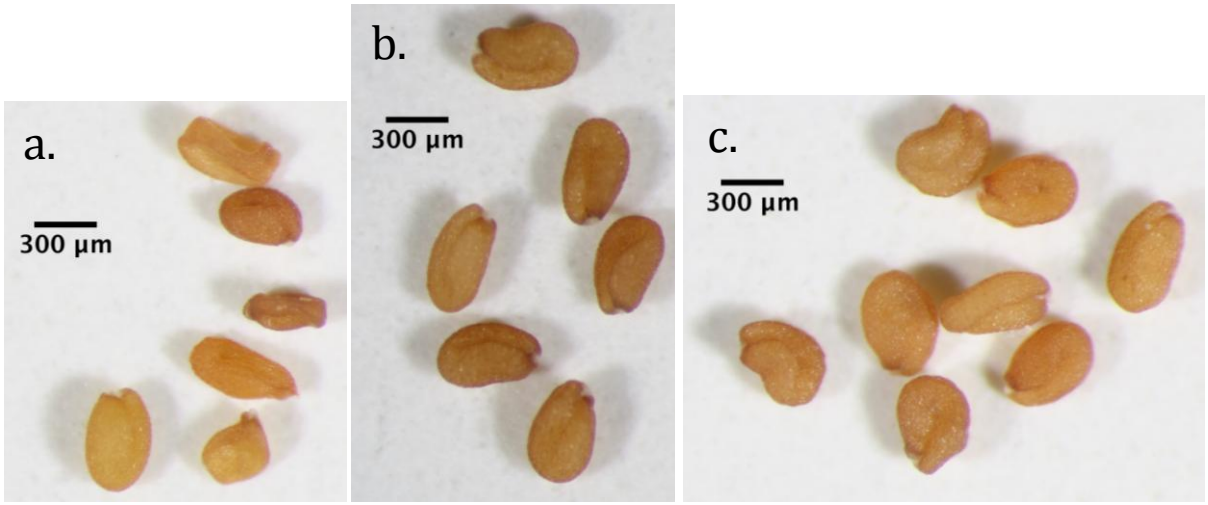


Figure 14: Seeds harvested from an *ijT1* plant grown at 12°C.

In the seeds grown at 22°C, no difference in appearance of the genotypes was apparent. However, when looking at the seeds harvested from plants grown in 12°C, there seems to be a bend in some *ijT1* seeds that is not seen in the WT seeds at 12°C.

DISCUSSION

When plants are grown in lower temperatures there is a decrease in performance of enzymes, and cellular processes can slow down or even halt completely. Temperate plants, like *Arabidopsis thaliana*, have developed a mechanism to help combat the harmful effects of the cold. Cold temperatures slow the growth rate of plants, even in cold-acclimated plants like *Arabidopsis thaliana*. This sluggish growth results from the slow functioning enzymes due to the cold. Even though the plants grow slower as a whole in the cold, there is a difference in growth rate between the wild type *Arabidopsis* and the *ijT1* mutant of *Arabidopsis*, as seen in **Figure 2** and **Figure 3**. This suggests that there must be an aspect of the *IOJAP* gene that is temperature dependent or important for cold acclimation.

The phenotype of the leaves in low temperatures was different than that of the leaves in normal temperatures (22℃). We saw a color change in the true leaves of *Arabidopsis* plants at lower temperatures. The phenotypic changes were manifest in the young leaves, and as the plants aged the differences became less significant. This could mean that the plant eventually cold-acclimated but initially they could not handle the cold as well as the wild type plants. When the true leaves appeared on all plants grown at 22℃ they were green, without excessive curling (**Figure 4**). However, when leaves first appeared on the *ijT1* plants grown in 12℃ they appeared to be less green in color and had a different shape about them. The true leaves were hyponastic, in a half-moon shape

(**Figure 5**). The color was not uniform and more yellow than green, suggesting there were fewer properly functioning chloroplasts and abnormal cellular growth.

Even though these plants grow at a much slower pace in 12℃ and 4℃ than they do at 22℃, that doesn't necessarily affect the overall size of the plant. There was no difference in the number of leaves present on the day of inflorescence emergence between WT and *ijT1* in 12℃. There was a difference in the rosette radii at lower temperatures. However, this difference was also present in the plants grown at 22℃, which suggests the radii may not be temperature dependent. The mutant *ijT1* plants had overall shorter inflorescence height in both 22℃ and 12℃ grown plants. It is possible that the IOJAP gene is important for proper and full growth of Arabidopsis plants even in normal temperatures (22℃).

When looking at seed size, the different mass of the seeds from *ijT1* and WT plants is interesting because even though they look the same on the outside, the mutant seeds harvested from plants grown in 12℃ have a higher density than those from WT plants in both small and medium seed sizes, but not in the large size. This could be due to the lack of data in the large seed category. Between WT and *ijT1* there were seven plants overall that did not produce at least 100 large seeds, so there are fewer data points in the large seed category than there are in the small and medium categories.

While not every phenotype explored in these experiments showed a difference between mutant *ijT1* plants grown in low temperatures (12℃ and 4℃) and those grown in normal temperatures (22℃), we do see changes in some such as root growth rate, leaf appearance and root length. These changes in the *ijT1 Arabidopsis* when exposed to cold temperatures suggest a mutation in the *IOJAP* gene affects cold acclimation.

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