5-2016

Low temperature phenotype in Arabidopsis thaliana mutant ijT1 is affected by splicing

Connie Liu
University of Tennessee, Knoxville, cliu39@vols.utk.edu

Low temperature phenotype in *Arabidopsis thaliana* mutant *ijT1* is affected by splicing

By

CONNIE LIU

Advisor: Andreas Nebenführ

May 2016

An Honors Thesis

Presented to the Faculty of the

Department of Biochemistry and Cellular and Molecular Biology

& the Chancellor's Honors Program

University of Tennessee, Knoxville

In Partial Fulfillment of the

Requirements for the Degree of

Bachelor of Science

ABSTRACT

Low temperatures negatively affect the quality and yield of plants. Many plants, including *Arabidopsis thaliana*, have evolved mechanisms that allow them to acclimate to cold temperatures, enhancing their tolerance to these low temperature conditions. Although wild type *Arabidopsis thaliana* plants can acclimate to 12 ℃ and 4 ℃, the *Arabidopsis* mutant *ijT1,* which is a mutation in the *IOJAP* gene that encodes a chloroplast protein, has difficulties acclimating to this drop in temperature. While the *ijT1* plant functions normally at 22 ℃, lower temperatures negatively affect its phenotype. Additionally, *ijT1* plants appear to recover over time as they mature at 12 ℃ but not at 4 ℃. IOJAP proteins are hypothesized to affect translation, as it appears that they have an impact on ribosome biogenesis and the formation of the large ribosomal subunit. There are at least two possible explanations for the temperature-sensitive phenotype of *A. thaliana*: protein targeting and splicing. *Arabidopsis thaliana* has 2 *IOJAP* genes, *cp-IOJAP* and *mt-IOJAP*, that are believed to encode proteins directed to the chloroplast and mitochondria, respectively. Protein import, however, is inhibited by low temperatures. Because the transit peptide sequence of *mt-IOJAP* appears to be dual targeted to the mitochondria and the chloroplast, we hypothesized that mt-IOJAP can compensate for the loss of cp-IOJAP in normal temperature environments in the mutant *ijT1* but not at low temperatures. Further work in the lab is needed before addressing any possible conclusions regarding this protein targeting process. Additionally, the *ijT1* plants have a T-DNA insertion in the first intron of the *cp-IOJAP* gene. This mutant will only be able to produce the wild type transcript if the intron with the inserted T-DNA sequence is properly spliced out. Exposure to cold stress affects splicing and may prevent the T-DNA insertion from being spliced out. We hypothesized that increasingly colder temperatures will lead to reduced splicing in mutant *ijT1* plants at low

temperatures because the T-DNA may be spliced out at 22 ℃ but not at 12 ℃ or 4 ℃. Our results suggested that the T-DNA is properly spliced out at 22 ℃, spliced out with a decreased efficiency at 12 ℃, and not spliced out at all at 4 ℃. The phenotype recovery of *ijT1* at 12 ℃ may occur as the amount of successfully spliced products gradually accumulates over time. We conclude that the splicing defect of the *ijT1* mutant may be able to explain the temperature sensitivity of the mutant.

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INTRODUCTION

The Arabidopsis thaliana mutant *ijT1* is negatively affected by lower temperatures

Cold affects the quality and yield of plants; because of this, it is important to understand how plants are able to respond and adapt to low temperatures (Hannah et al. 2005). Many plants, including *Arabidopsis thaliana*, have evolved mechanisms that enhance their tolerance to low, but non-freezing temperatures (Miura & Furumoto 2013). This process is referred to as cold acclimation. Cold acclimation consists of complex physiological and biochemical changes that affect growth and water balance, membrane and cell wall composition, and cold-regulated gene expression and protein levels (Hannah et al. 2005). Although wild type *Arabidopsis thaliana* plants can acclimate to 12 ℃ and 4 ℃, we have found an *Arabidopsis thaliana* mutant, *ijT1,* that has trouble acclimating to this drop in temperature. The chloroplast function of *ijT1* plants appears to be impacted by changes in temperature, as their phenotypes are exacerbated at increasingly lower temperatures. While *ijT1* plants are able to function normally at 22 ℃, previous work in the lab showed that lower temperatures cause *ijT1* plants to be smaller and exhibit abnormal leaves and roots that are approximately 60-80% shorter than those of the wild type plant. When exposed to cold, these leaves become chlorotic, have protruding hydathodes, and show the hyponastic response (**Figure 1**). These leaves, however, appear to recover over time as they mature at 12 ℃ but remain chlorotic at 4 ℃. Additionally, the phenotype seems to be most apparent in young developing leaves. There are at least two possible explanations for the temperature-sensitive phenotype of *A. thaliana*: protein targeting and splicing.

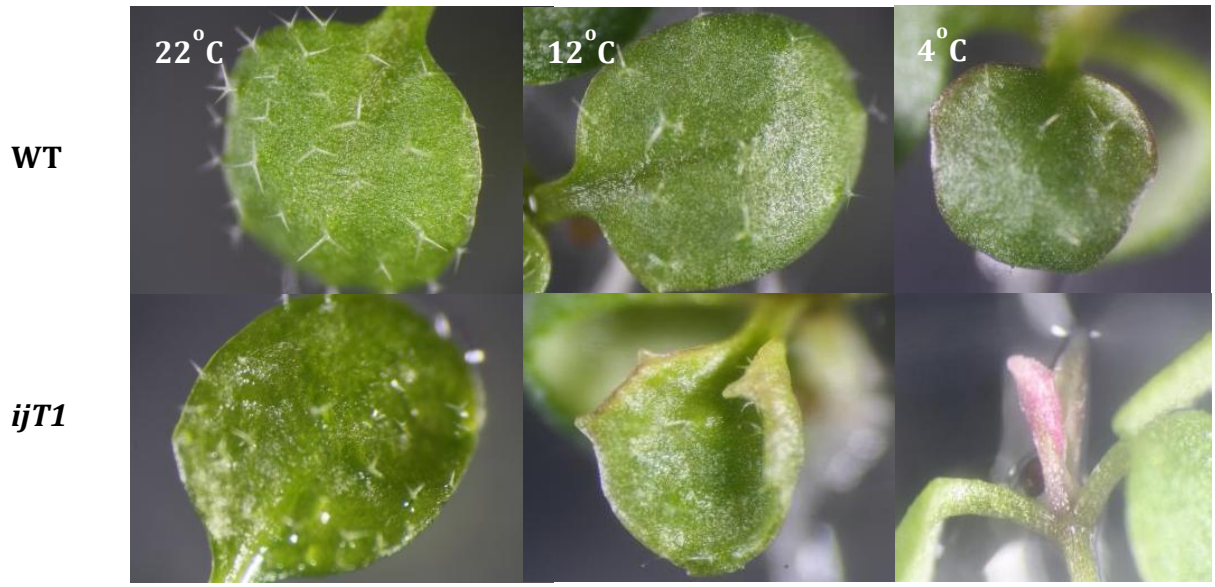

Figure 1. The effect of increasingly cold temperatures on *Arabidopsis thaliana* wild type and mutant *ijT1.* All pictures taken at the same magnification. When exposed to cold, the leaves of the mutant ijT1 are smaller than those of the wild type. They become chlorotic, have protruding hydathodes, and show the hypnoastic response.

Transit peptide sequences of IOJAP proteins in *Arabidopsis* direct protein import

Arabidopsis thaliana has two homologous IOJAP proteins. Although these proteins are widely conserved among different organisms, their function remains poorly characterized (Butland et al. 2015). Based on the function of IOJAP orthologues in *Escherichia coli (E. coli)* and human mitochondria, *IOJAP* genes in *Arabidopsis* are suspected to affect protein translation, as they are associated with ribosome biogenesis and the formation of the large ribosomal subunit (Häuser et al. 2012; Fung et al. 2013). The *Arabidopsis* mutant *ijT1* is a mutation in the IOJAP gene directed toward the chloroplast. These *IOJAP* genes are located in the nucleus and their encoded proteins are suspected to be imported to the mitochondria or chloroplast via transit peptide (TP) sequences that act as signals attached to the IOJAP protein. The TP sequence is read post-translationally and directs the IOJAP protein to the organelle of its final destination. The TP sequences of genes that are imported in the mitochondria generally have an abundance of

positively charged arginine residues and have the capacity to form amphipathic alpha helices (Ge et al. 2014). The TP sequences of genes that are targeted to the chloroplast, on the other hand, have an abundance of serine and proline and are generally longer and unstructured (Ge et al. 2014). There are also transit peptides that are dual targeted to both the mitochondria and chloroplasts; these use an ambiguous dual targeting peptide that has an intermediate sequence pattern between that of the chloroplast and mitochondria (Ge et al. 2014).

The *mt-IOJAP* gene (At1g67620) is hypothesized to encode a protein that is transferred to the mitochondria based on tandem mass spectrometry (MS/MS) (Hooper et al. 2014). MS/MS also determined that the cp-IOJAP protein (At3g12930) likely localizes to the chloroplast (Hooper et al. 2014). Additionally, Target P analysis of the TP sequences indicated that there was a 91.5% probability that the cp-IOJAP protein would be translocated to the chloroplast. The mt-IOJAP protein was found to have a 66.7% chance of being translocated to the mitochondria and a 57.6% chance of being translocated to the chloroplast (Emanuelsson et al. 2007), suggesting that it may be dual targeted to both organelles (**Figure 2**). The *A. thaliana* mutant *ijT1* is a mutation of *cp-IOJAP*, as it has an insertion in the first intron of the *cp-IOJAP* gene. At 22 °C, with a mutant *ijT1* plant, we hypothesize that the mt-IOJAP protein can be imported to the chloroplast to rescue the lack of cp-IOJAP of the *Arabidopsis* plant. At 12 °C, however, it is possible that the mt-IOJAP protein cannot be transported to the chloroplast efficiently, impairing the chloroplast function of the *A. thaliana ijT1* plant. This import occurs across organelle membranes and has been reported to be inhibited by low temperatures (Leheny & Theg, 1994). For example, the effect of temperature on protein translocation has been noted in chloroplasts, as reactions that occur rapidly at 25 °C are significantly slowed at lower temperatures (Leheny & Theg, 1994). By observing the cp-IOJAP and mt-IOJAP proteins of *A. thaliana* and their

subcellular localization, we plan to address whether or not mt-IOJAP can compensate for the loss of cp-IOJAP in normal temperature environments.

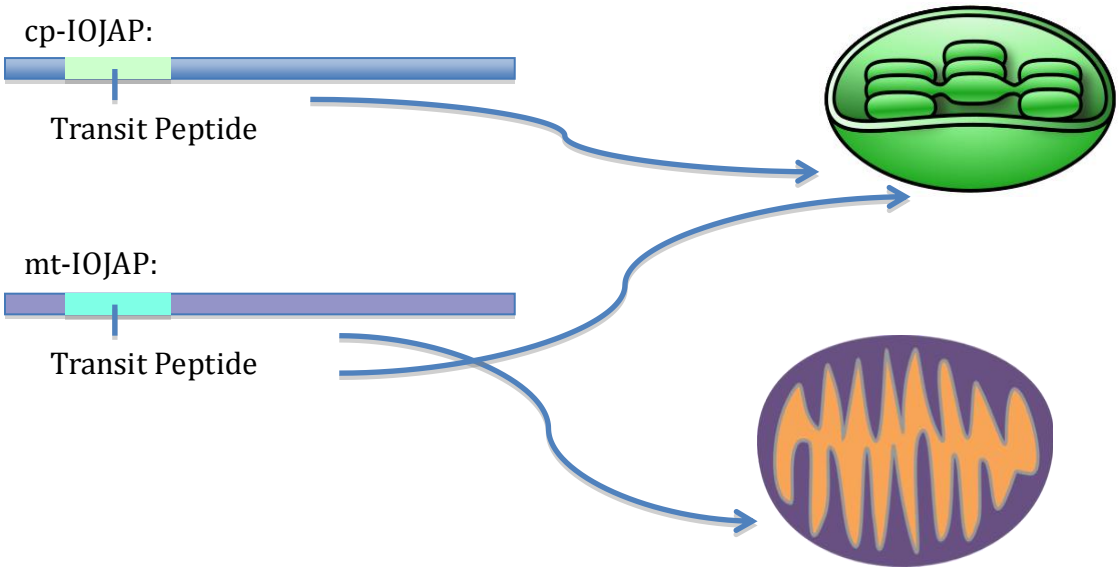

Figure 2. The cp-IOJAP and mt-IOJAP proteins are directed to their final destinations by transit peptides. While it is very likely that the cp-IOJAP protein is translocated to the chloroplast (91.5%), the translocation of the mt-IOJAP protein is predicted to be split between the mitochondria (66.7%) and the chloroplast (57.6%) (Emanuelsson et al. 2007).

Cold stress affects splicing in *Arabidopsis*

The *ijT1* plants have a transfer DNA (T-DNA) insertion in the first intron of the *cp-IOJAP* gene (**Figure 3**). Most T-DNA insertions lead to loss-of-function alleles; for example, if the mRNA is transcribed with the T-DNA insertion, the T-DNA sequence may contain stop codons that would result in premature translation termination (Wang 2008). Wang (2008) found that when T-DNA is inserted in an intron, it does not have an effect on the transcript level in only 0.7% of cases. When the T-DNA is inserted into an intron, the mutant will be able to produce the wild type transcript only if the intron with the inserted T-DNA sequence is spliced out (Wang 2008). Although wild type transcripts may be produced, they are often made with decreased efficiency and a reduced level of the correctly spliced transcript (Wang 2008). RNA splicing

works to remove introns and join exons together and is required before mRNA can be translated into a protein.

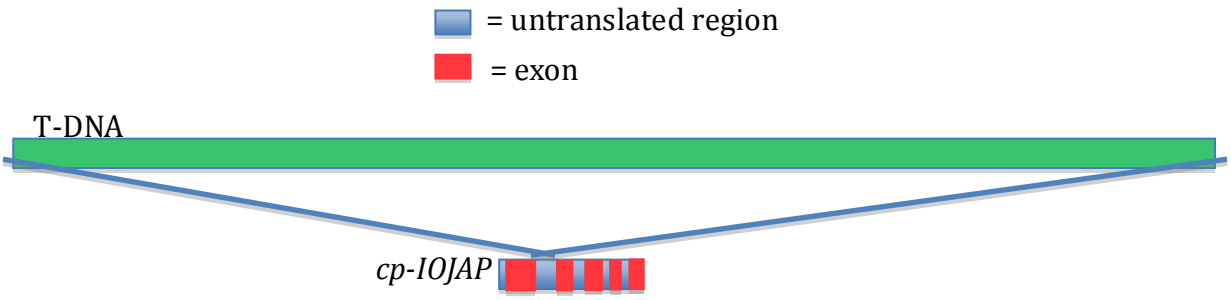

Figure 3. Arabidopsis thaliana ijT1 has a T-DNA. The T-DNA insertion is approximately 10,000 bp long and located in the first intron of the *cp-IOJAP* gene. Figure drawn using scale 1 in = 1000 bp.

Splicing plays an important role in post-transcriptional gene regulation, as it contributes to proteome diversity and can affect mRNA stability (Leviatan et al. 2013). Exposure to biotic and abiotic stresses affects splicing (Staiger 2015); cold stress, for example, affects the splicing of precursor-mRNA (pre-mRNA) and results in a change in splicing patterns (Leviatan et al. 2013). Increasingly colder temperatures may affect whether or not the first intron of the *cp-IOJAP* gene (and the T-DNA inserted in that intron) is spliced out. We hypothesized that increasingly colder temperatures have a greater effect on the low temperature phenotype of mutant ijT1 plants because the T-DNA may be spliced out at 22 °C in the ijT1 plant but not at 12 °C or 4 °C.

MATERIALS AND METHODS

Amplification of Insert

To amplify the *cp-IOJAP* sequence present in the wild type *Arabidopsis thaliana* plant, polymerase chain reaction (PCR) was performed on genomic DNA (gDNA) extracted from the wild type plant. Phusion DNA Polymerase was used because it is a high fidelity enzyme that has the lowest probability for mistakes. The Phusion protocol was followed as described by New England Biolabs (NEB) with the following primers and annealing temperatures (**Table 1**). The primers were designed to have SpeI and XmaI restriction enzyme sites (underlined).

Table 1: PCR to amplify the cp-*IOJAP* sequence

DNA sequence	Primer sequence	Annealing temperature (°C)	Extension time (min)
cpIJ-cI-F1	5'-CGAACTAGTAGAACAAA GGCGAAGGGTTTTAGAT -3'	69	1:30
cpIJ-cI-R1	5'-GCACCCGGGGTTCCGTGG TTGTGACTGATCC-3'		

Isolation of plasmid

Cultures of *Escherichia coli (E. coli)* bacteria were grown in LB liquid media with 100 µg/mL of ampicillin to isolate plasmid pAN578. This plasmid has the amp^R gene that confers resistance to the antibiotic ampicillin and a sequence encoding a cerulean fluorescent protein (CFP). pAN578 also has restriction enzyme sites that can be cut by the restriction enzymes SpeI and XmaI. These restriction enzyme sites are located between the promoter sequence (*35S*) and the *CFP* sequence of the plasmid. Additionally, the SpeI and XmaI restriction enzyme sites are also present on the PCR product of the *cp-IOJAP* gene, which can be inserted into the plasmid vector.

Restriction digest and ligation

A double digest was performed using SpeI and XmaI for both the *cp-IOJAP* PCR product and the pAN578 plasmid. A solution was prepared with 10 units SpeI, 10 units XmaI, 10x Cutsmart NEBuffer, and 1 µg DNA for each digestion. These enzymes have 100% activity in the 1X Cutsmart NEBuffer. The remaining volume of the digest consisted of dH_20 to bring the total reaction volume to 20 µL. The digestion was incubated for 2 hours at 37 °C. The Wizard SV Gel and PCR Clean-Up System was then used on the digested *cp-IOJAP* insert and pAN 578 plasmid products.

Following digestion, a ligation reaction involving a 3:1 insert to plasmid molar ratio (19.15 ng insert: 25 ng plasmid) was performed to place the insert in the plasmid. The ligation reaction took place at 14 °C for 24 hours and was then held at 4 °C. The plasmid products were transformed using Top10 *E. coli* competent cells by heatshock. 100 µL of these transformed cells were spread on ampicillin resistant plates (LB and 100 µg/mL ampicillin) and incubated for 14 hours.

Colony PCR

Following incubation, colony PCR was performed to search for plasmids with the appropriate insert. Each PCR reaction was performed with a small sample obtained from a single colony, Taq DNA polymerase, and the following primers and annealing temperatures (**Table 2**). Gel electrophoresis was performed with 1% agarose to determine which colonies contained transformed plasmids.

Table 2: Colony PCR of transformed DNA

Primer	Primer sequence	T_m (°C)	Extension time (min)
35S-F	5'-CGCACAATCCCACTATCCTTCGCA-3'	60	2:00
CFP-R	5'-CTGCACGCCCCAGGTCAG-3'		

Amplification of transformed plasmid

The colonies of the successfully transformed plasmids were grown in LB liquid media with 100 µg/mL of ampicillin at 37 °C. The new plasmids were sequenced at the UT Molecular Biology Resource Facility with the following primers (**Table 3**) to determine whether or not the correct insert was placed in the plasmid vector. These sequences were analyzed using Sequencher and Meg Align from DNAStar.

Table 3: DNA sequencing

Primer	Primer sequence
35S-F	5'-CGCACAATCCCACTATCCTTCGCA-3'
CFP-R	5'-CTGCACGCCCCAGGTCAG

TA cloning: Amplification of insert

The cp-*IOJAP* sequence was amplified as mentioned above. Following the initial PCR, an additional PCR was performed by adding Taq polymerase to the Phusion PCR product. This generated a single 3'-adenosine overhang at the end of each strand of DNA.

Cultures of *Escherichia coli* (*E. coli*) bacteria were grown in LB liquid media with 100 µg/mL of ampicillin to isolate plasmid pKRX. This plasmid was digested with the enzyme XcmI to leave a thymine residue at each end of the vector. This allows the insert and vector to hybridize due to the complementarity of their adenine and thymine nucleotides. A ligation reaction involving a 3:1 insert to plasmid molar ratio (30 ng insert: 25 ng plasmid) was performed to place the insert in the plasmid. The ligation reaction took place for 24 hours, continually cycling through the following temperatures in ten minute intervals: 16° C, 12° C, 8° C, and 4° C. (Matsumura 2015). The plasmid products were then transformed into Top10 *E. coli* competent cells by heatshock. 100 µL of these transformed cells were spread on ampicillin

containing plates (LB and 100 μg/mL ampicillin) and incubated for 14 hours. Following incubation, colony PCR, with the primers listed in Table 4, was performed to search for plasmids with the appropriate insert.

Table 4: Colony PCR of transformed DNA (pKRX + *cp-IOJAP*)

Primer	Primer sequence	T_m (°C)	Extension time (min)
cpIJ-cl-F1	5'-CGAACTAGTAGAACAAA GGCGAAGGGTTTTAGAT -3'	53	0:30
cp-TP20-R	5'-GCATCTAGACTCATTC GCTCACATTC- 3'		

RNA extraction and Reverse Transcriptase PCR

Wild type and mutant *ijT1 A. thaliana* seeds were sterilized, stratified, and held at 4 °C for two days. The seeds were then plated on MS plates (1/2 MS, 1% sucrose, 5% phytagel) and held at 22 °C for two days before being moved to their target temperatures. Wild type and mutant *ijT1 A. thaliana* seeds were grown at 22° C for 10 days, 12° C for 42 days, and 4° C for 61 days. RNA extraction was then performed by freezing the roots and leaves of the plants with liquid nitrogen and homogenizing them with a mortar and pestle and 500 μL TRIzol (Life Technologies) per 50 mg tissue. The RNA was precipitated, washed, and treated with DNase I. The extracted RNA was used to synthesize cDNA with M-MuLV Reverse Transcriptase (New England BioLabs). A PCR was performed on the cDNA using three sets of primers (**Table 5**). Gel electrophoresis was performed to compare the lengths of the products generated with the anticipated product lengths (**Table 6**).

Table 5: PCR of cDNA

Primers	Primer sequences	T_m (°C)	Extension time (min)	Cycle #
mt-TP20-F1 + mt-cl-R1	5'-CGATCTAGACTCGACGTGAAAGCTGA-3' + 5'GCACCCGGGAGAATAGCCCGGTTTCCT-3'	56	1:00	40
Rv-ij-LB + Rv-iojap	5'-CCTTATAATTGCTGTTGGCGACT-3' + 5'-TCTCTGCATCATCGTCAACC-3'	54	0:45	40
cp-TP20-F + cp-cl-R1	5'-CGATCTAGAGAGAGACTTGTTCAACA-3'+ 5'-GCACCCGGGGTTCCGTGGTTGTGACTGATCC-3'	56	1:00	40

Table 6: Anticipated PCR products

Primers	Product size (bp)	Length of gDNA (bp)
mt-TP20-F1 + mt-cl-R1	450	~1000
Rv-ij-LB +Rv-iojap	223	~300
cp-TP20-F + cp-cl-R1	515	~950

RESULTS AND DISCUSSION

Efficiency of Chloroplast Import at Low Temperatures

mt-IOJAP has previously been inserted into the plasmid pAN581, which encodes a yellow fluorescent protein (YFP) using the SpeI and XmaI restriction enzyme sites. Following successful insertion of *cp-IOJAP* into the pAN578 plasmid, both tagged proteins will be bombarded into plant cells using tungsten particles coated with the plasmid DNA. These particles will be shot into the cells in the epidermal strip of an onion storage leaf. The location of the fusion proteins encoded by the plasmids will be tracked by the fluorescent proteins (CFP or YFP) present in their respective coding sequences using an inverted fluorescent light microscope (Zeiss). The fluorescent proteins are able to track the movement of these proteins and thus

determine the destination of the proteins encoded by *cp-IOJAP* and *mt-IOJAP* genes. This particle gun bombardment will be performed at 22 °C, 12 °C, and 4 °C to see which organelle(s) receives the cp-IOJAP and mt-IOJAP transformed proteins at each temperature. This will be determined by using microscopy.

To create the cp-IOJAP-CFP fusion protein, we inserted the genomic sequence *of cp-IOJAP* into the pAN578 plasmid. Colony PCR was performed to determine whether or not the insert DNA was present in the plasmid. Gel electrophoresis followed to analyze the size of the DNA in the colonies. The plasmid pAN 578 showed a band at ~ 300 base pairs, as PCR involved part of the *35S* promoter and the *CFP* sequence. The plasmid pAN 578 with a *cp-IOJAP* insert is expected to show a band at ~1500 base pairs because the restriction enzyme sites for SpeI and XmaI lie between the *35S* promoter sequence and the fluorescent protein **(Figure 4)**. This ~1500 base pair sequence would indicate that the 1234 base pair cp-*IOJAP* insert has been successfully integrated into the pAN 578 plasmid between the restriction enzyme sites.

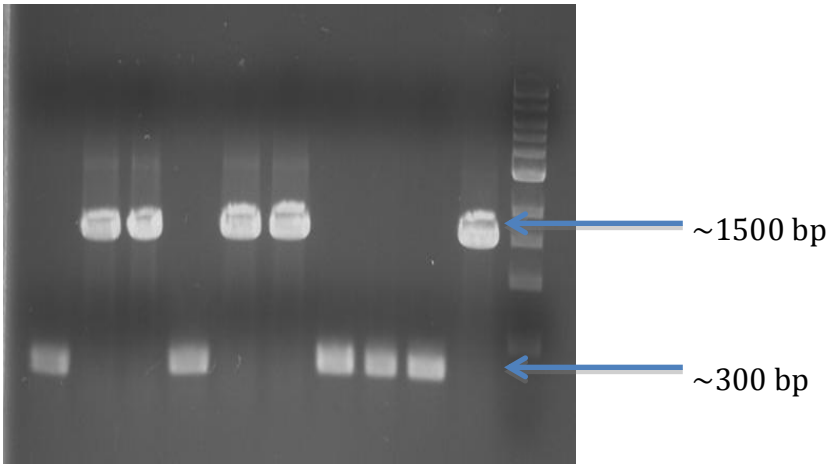

Figure 4. Colony PCR of ligated pAN578 and *cp-IOJAP.*
1% gel ran at 100 Volts for 30 min.

DNA collected from colonies that appeared to be successful ligations was then amplified and sequenced to determine the precise order of nucleotides within the DNA. Sequencher was

used to compare the DNA sequence of the transformed pAN 578 vectors with the *cp-IOJAP* gene of *A. thaliana*. The sequence of the DNA using the forward primer 35S is represented by the top two lines of Figure 5 (Q101_E11_cp-igap-578#3_Seq35s-F1_044, Q101-G11-cp-igap-578#6_Seq35s_F1_042), as two samples were sequenced. The reverse primer CFP-R was also used to sequence the DNA, which is shown on the bottom two lines of the figure (Q101_F11_cp-igap-578#3-Seq-CFP-RQ_043, Q101_H11_cp-igap-578#6-Seq-CFP-RQ_041). These sequences were compared against the sequence of the *cp-IOJAP* insert, which is also known as *cp-IOJAP* gDNA TAIR. Although the sequence of the cp-*IOJAP* insert in the pAN 578 plasmid appeared to be the correct number of base pairs in length, a deletion was detected in the reverse iojap primer (cpIJ-cI-R1) when compared to the cp-*IOJAP* gene **(Figure 5**). This deletion results in a frameshift mutation that would prevent translation of the *CFP* sequence of the plasmid.

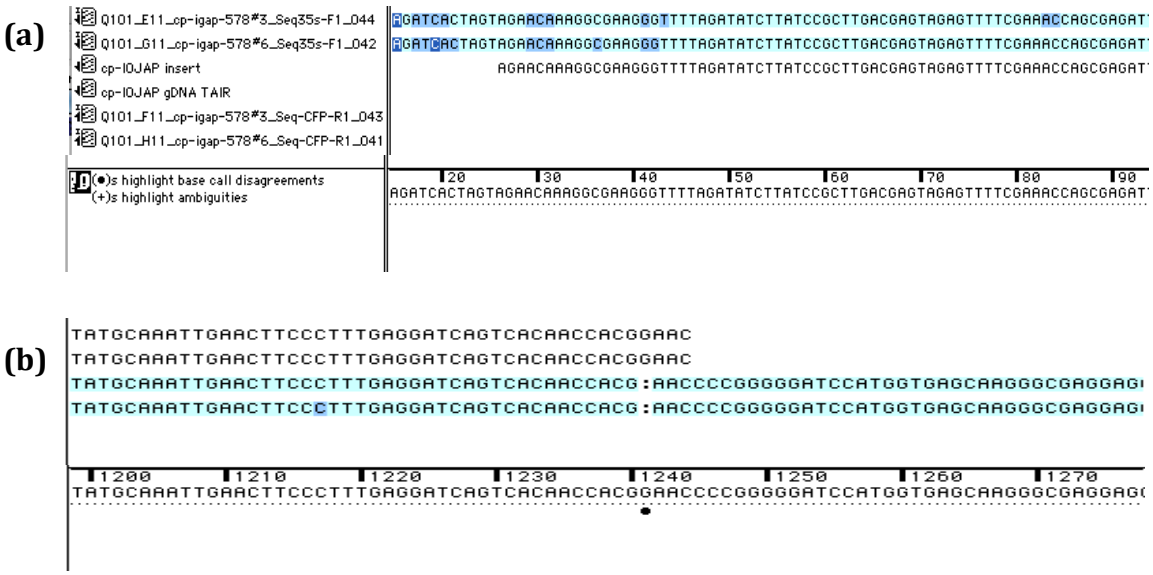

Figure 5. (a) DNA sequencing of two vectors transformed with cp-IOJAP of A. thaliana and compared to the cp-IOJAP insert sequence. **(b).** At ~1240 bp, a base pair deletion is seen in the reverse primer.

Because there was a deletion in the reverse primer used to amplify the *cp-IOJAP* sequence (cpIJ-cl-R1), new primers were ordered and the cloning experiment was repeated. Because the *cp-IOJAP* product was generated with primers designed with the SpeI and XmaI

restriction enzyme sites, these sites are located at the far ends of the *cp-IOJAP* sequence. It was impossible to distinguish between the digested and undigested *cp-IOJAP* product on the gel, as there was only an 8 base pair difference in size between the digested and undigested product. To ensure that only the digested *cp-IOJAP* insert product is being used in the ligation, the *cp-IOJAP* PCR product was first inserted into the pKRX plasmid by TA cloning. This would allow us to easily distinguish between the digested product and any products that did not successfully complete the double digest reaction. Colony PCR was performed following TA cloning to determine whether or not the ligation of the pKRX plasmid and cp-*IOJAP* was successful. The transformation reaction yielded one colony and gel electrophoresis was used to analyze the DNA in that colony. A band is shown at ~500 bp, which was the expected product length given the chosen primers (**Figure 6**). In a next step, this transformed product will be digested alongside the plasmid pAN578 with the restriction enzymes SpeI and XmaI. A ligation reaction will then be performed with these digested products to clone the *cp-IOJAP* gene in a plasmid with *CFP*.

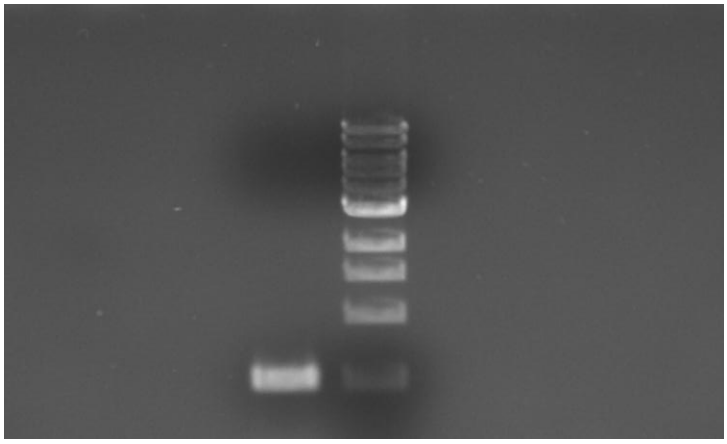

Figure 6. Colony PCR of ligated pKRX and *cp-IOJAP*. 1% gel ran at 100 Volts for 30 min.

Efficiency of Splicing at Low Temperatures

Because it has been found that a mutant will only be able to produce the wild type transcript if the intron with the inserted T-DNA is spliced out (Wang 2008), gel electrophoresis of the RT-PCR products was performed to compare the products of *ijT1* with those of the wild type. This was done to verify whether or not the T-DNA present in the first intron of the mutant *ijT1* was spliced out at 22 °C, 12 °C, and 4 °C. Table 5 lists the three primer sets used throughout the experiment. Primer set 1 (mt-TP20-F1 + mt-cl-R1) serves as a control that amplifies a DNA sequence in the *mt-IOJAP* gene. DNA should be amplified in both the wild type and mutant plants. Primer set 2 (Rv-ij-LB + Rv-iojap) and primer set 3 (cp-TP20-F + cp-cl-R1) amplify sequences in the *cp-IOJAP* gene. The forward and reverse primers in primer set 2 anneal on the first and second exon (**Figure 7**); a product should only be generated if the T-DNA is properly spliced out. The primers of primer set 3 anneal on the second exon and the last exon and may also be produced if the T-DNA is not spliced out (**Figure 7**).

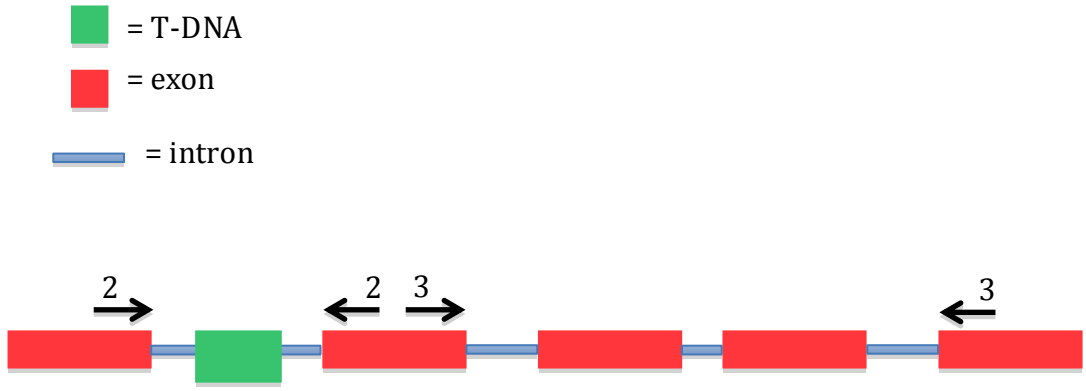

Figure 7. Location of primer sets 2 and 3 on the cp-IOJAP gene. Not drawn to scale.

Gel electrophoresis showed that the products were the expected length (**Figure 8**). DNA from *A. thaliana* grown at 22 °C appeared similar for both the wild type and *ijT1* plants. The bands for the *ijT1* plants grown at 12 °C were not as bright as those of the wild type, which is indicative of decreased DNA amplification in the *ijT1* plants. These results may suggest that a decrease in splicing efficiency occurs at 12 °C. The phenotype recovery of *ijT1* at 12 °C may

occur as the amount of successfully spliced products increases over time. The greatest effect is seen with plants grown at 4 °C. Primer set 1 shows no effect on the *ijT1* plants grown at 4 °C, as this primer set relied on cDNA sequences from a control gene (*mt-IOJAP)* that is not affected by the T-DNA insertion. When primer set 2 was used on *ijT1* grown in 4 °C, no DNA was amplified. Additionally, when primer set 3 was used on *ijT1* grown in 4 °C, the amount of DNA that was amplified was noticeably less than that of the wild type. These results show that T-DNA is likely spliced out at 22 °C and even 12 °C, but not at 4 °C.

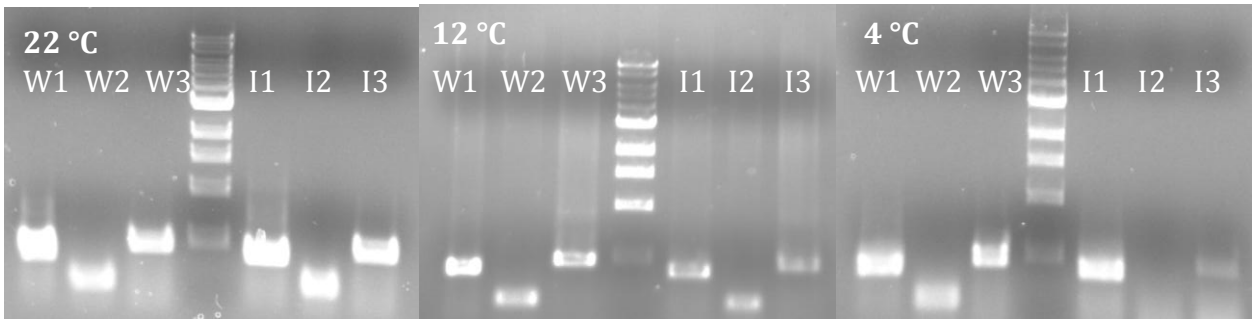

Figure 8. PCR of cDNA. 1% gels ran at 100 Volts for 30 min. W= wild type, I= *IjT1*

CONCLUSIONS

We hypothesized that at 22 °C, with a mutant *ijT1 A. thaliana*, the mt-IOJAP protein can be imported to the chloroplast, which is why these plants appear healthy. At 12 °C and 4 °C, however, it is likely that the mt-IOJAP protein cannot be transported to the chloroplast to compensate for the loss of the cp-IOJAP. Difficulties in assembling the *cp-IOJAP*-CFP plasmid prevented us from testing this hypothesis. Once this construct has been created, particle bombardment of mt-IOJAP and cp-IOJAP will allow us to track the subcellular localization of these proteins to determine whether or not lower temperatures affect protein import.

We also hypothesized that increasingly colder temperatures negatively affect the low temperature phenotype of mutant *ijT1* plants because although the T-DNA may be spliced out of

ijT1 at 22 °C, this may not occur at 12 °C or 4 °C. Our results suggest that the T-DNA is properly spliced out at 22 °C, spliced out with a decreased efficiency at 12 °C, and not spliced out at all at 4 °C. A splicing defect at low temperatures may explain the phenotype of the mutant *ijT1* plants. Comparisons of the gels of wild type and *ijT1* plants show that DNA amplification is unaffected at 22 °C but appears to decrease with increasingly colder temperatures in *ijT1* in comparison to the wild type plant. At 12 °C, it appears that a decrease in splicing efficiency occurs, as some products may be properly spliced while others are not. These results, however, are not quantitative so the wild type and *ijT1* template levels will need to be investigated in more detail. The amount of DNA throughout PCR must also be further explored to ascertain that DNA amplification was being observed prior to saturation of the PCR product. The PCR that was performed on the cDNA is only able to give results of DNA amplification after the PCR has completed all of its cycles; this cycle number may have been too large to reveal differences in template concentration. Because the PCR products of the cDNA sequences generated from RT PCR are the expected length, qPCR will be performed to amplify the DNA. qPCR uses fluorescent dyes to label PCR products during thermal cycling to give precise quantification of the PCR products. This would show the amount of DNA produced at each time point, which would show whether or not increasingly colder temperatures affect splicing of the DNA in the mutant *ijT1*.

REFERENCES

G. Butland, J.M. Peregrín-Alvarez, J. Li, W. Yang, X. Yang, V. Canadien, A. Starostine, D. Richards, B. Beattie, N. Krogan, et al. (2005). **Interaction network containing**

conserved and essential protein complexes in Escherichia coli. *Nature* 433, 531-537.
O. Emanuelsson, S. Brunak, G. von Heijne, H. Nielsen (2007). **Locating proteins in the cell using TargetP, SignalP, and related tools**. *Nature Protocols* 2:953-971.
C. Ge, E. Spanning, E. Glase, A. Wieslander (2014). **Import Determinants of Organelle Specific and Dual Targeting Peptides of Mitochondria and Chloroplasts in Arabidopsis thaliana**. *Molecular Plant* 7.1:121-136.
S. Fung, T. Nishimura, F. Sasarman, and E.A. Shoubridge. (2013). **The conserved interaction of C7orf30 with MRPL14 promotes biogenesis of the mitochondrial large ribosomal subunit and mitochondrial translation.** *Mol Biol. Cell* 24, 184-193.
M.A. Hannah, A. G. Heyer, D. K. Hincha (2005). **A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana***. PLoS Genet 1(2):e26.
R. Häuser, M. Pech, J. Kijek, H. Yamamoto, B. Titz, F. Naeve, A. Tovchigrechko, K. Yamamoto, W. Szaflarski, N. Takeuchi, et al. (2012). RsfA (YbeB) **Proteins are Conserved Ribosomal Silencing Factors.** *PloS Genet*. 8, e10002815.
C. M. Hooper, S. K. Tanz, I. Castleden, M. Vacher, I. Small; A. H. Millar (2014) **SUBAcon: a consensus algorithm for unifying the subcellular localization data of the Arabidopsis proteome.** *Bioinformatics*. 30(23):3356-64 (Bioinformatics.) (PubMed)
E. A. Leheny, S. M. Theg (1994). **Apparent Inhibition of Chloroplast Protein Import by Cold Temperatures Is Due to Energetic Considerations Not Membrane Fluidity.** *The Plant Cell* 6:427-437.
N. Leviatan, N. Alkan, D. Leshkowitz, R. Fluhr (2013). **Genome-Wide Survey of Cold Stress Regulated Alternative Splicing in *Arabidopsis thaliana* with Tiling Microarray.** *PLoS ONE* 8(6): e66511. Doi:10.1371/journal.pone.0066511.
I. Matsumura (2015). **Why Johnny can't clone: Common pitfalls and not so common solutions.** *BioTechniques* 59:IV-XII.
K. Miura, T. Furumoto (2013). **Cold Signaling and Cold Responses in Plants**. *International Journal of Molecular Science*. 14:5312-5337.
D. Staiger (2015). **Shaping the Arabidopsis Transcriptome through Alternative Splicing.** *Advances in Botany.* 2015. doi:10.1155/2015/419428
Y. H. Wang. **How effective is T-DNA insertional mutagenesis in Arabidopsis?** *Journal of Biochemical Technology*. 1(1):11-20.