Regulation of a Temperature-Induced Lipocalin (TIL) by Cold Stress and Epibrassinolide in the Gametophytes of *Ceratopteris richardii*

ABSTRACT

Global warming has been linked to many of the emerging weather patterns i.e. hotter heat waves, drier droughts, greater snowfall, etc. Numerous studies have shown that sudden extreme cold stress can significantly decrease crop productions. To develop crops that are capable of adapting to sudden temperature change, it is important to identify genes and signaling pathway specifically affected. This study identifies and characterizes the regulation of a Temperature Induced Lipocalin (TIL) gene in the semi-aquatic subtropical fern *Ceratopteris richardii*.

When gametophytes of *Ceratopteris* are treated with cold stress (4-10 ^{o}C), there was a significant reduction in the prothalli area and cell count, but an increase in cell elongation. This increase in cell elongation may be responsible for inflation or ballooning of the cells of the prothalli not observed at room temperature. When gametophytes however are pre-treated with 24-epibrassinolide (EBL), these inflations were dramatically reduced during cold stress, suggesting that brassinosteroid signaling pathway may be involved in mediating cold stress response. EBL pretreatment also allowed for the development of sporophytes, which were significantly reduced by cold stress.

Using various bioinformatics programs, an orthologue of a Temperature Induced Lipocalin (CrTIL) gene was identified and characterized from an EST cDNA library in *Ceratopteris richardii*. RT-PCR revealed that cold stress as well as the brassinosteroid signaling pathway increased the expression of the CrTIL gene.

Further characterization of CrTIL gene and the brassinosteroid signaling pathway in gametophytes can help develop strategies to allow plant to cope with sudden cold stress.

RESEARCH PROBLEM

How does cold stress due to climate change affect the expression of Temperature-Induced Lipocalin (TIL) in plants?

HYPOTHESIS

The putative orthologue of TIL1 found in *Ceratopteris richardii* will be upregulated under cold stress conditions (4-10 ^{o}C) relative to the spores treated under room temperature conditions. Furthermore, TIL will be upregulated when the spores are treated with 24-epibrassinolide (EBL) relative to the control group.

INTRODUCTION

Climate change is a prominent worldwide phenomenon, and it's already shown to have devastating implications on life. However, the effects are most severe on crops and farms. Extreme cold weather is able to facilitate plant death by freezing the cells of the plant when temperatures are below freezing point (Pennisi *et. al*, 2017). Furthermore, the plant's metabolism rates will be substantially reduced because enzymes, proteins and other important cellular functions will not be occurring in optimal temperature (Pennisi *et. al*, 2017). With the effects of climate change, extreme cold weather is occurring more frequently because of a phenomenon known as the polar vortex (Siegel, 2019). When there are warmer land temperatures, more heat is transported into the Arctic stratosphere, where cold masses of air is held together by these polar vortices. This destabilizes the polar vortex, thus bringing down the cold air and causing extreme cold events (Siegel, 2019). In fact, just last year - January to February 2019 - a severe polar vortex occurred in the U.S.; the Midwest was hit the hardest, and it experienced wind chills that were as low as -46^{o}C (BBC News, 2019). Consequently, the crop yield of farms and agricultural

facilities will take the biggest hit. In fact, studies have shown that extreme cold events have already caused significant damage on crop yields. For example, a study that was conducted in China indicated that extreme spring cold spells (ESCS) are having an increasingly profound impact on wheat yields (Chinese Academy of Sciences, 2018). When an ESCS occurs over North China, continuous negative temperature anomalies can induce wheat yield losses of up to 20 percent or more (Chinese Academy of Sciences, 2018). Thus, it is clear that with the increasing prevalence of extreme cold weather events, crops will have an increasingly difficult time in adapting to such extreme changes in the climate. Therefore, a more substantial mechanism for defending plants against extreme cold must be developed.

The Temperature Induced Lipocalin (TIL) is a membrane-associated protein that belongs to a larger family of lipocalins well known for their protective roles in mitigating temperature stress, drought, and salinity stresses. In addition to this, they have essential roles in the modulation of cell growth, induction of apoptosis and the regulation of immune response (Charron, Jean-Benoit Frenette, 2002 et al). Several studies in *Arabidopsis* have shown that TIL plays a role in basal and acquired thermotolerance salt stress as well as drought and high light stresses (Hernández-Gras, Francesc, *et. al*, 2015). Furthermore, in recent studies conducted by Hernández-Gras, Francesc, *et. al*, 2015 and Charron, Jean-Benoit Frenette, *et. al*., 2005, true plant lipocalins have been found in *Arabidopsis thaliana*, and *Triticum aestivum* (wheat), designated AtTIL and TaTIL respectively.

The structure of lipocalins are highly symmetrical, being dominated by a single eight stranded antiparallel beta sheet which closes back onto itself, forming a continuously hydrogen-bonded beta barrel (Charron, Jean-Benoit Frenette, *et al*., 2005). This barrel encloses a ligand-binding site composed of both an internal cavity and an external loop scaffold. The diversity of

the cavity and scaffold can allow for many different ligands to bind (Charron, Jean-Benoit Frenette, *et al*., 2005). These are generally small hydrophobic ligands such as retinoids, fatty acids, steroids, odorants, and pheromones. Furthermore, the 3D structure of lipocalins are highly conserved, containing three structurally conserved regions (SCRs), SCR-1, SCR-2 and SCR-3 (Charron, Jean-Benoit Frenette, *et al*., 2005).

The interaction of TIL with cell membranes is unique because this protein does not contain a recognizable signal for membrane targeting. Furthermore, TILs do not contain hydrophobic regions that have the features that follow a transmembrane pattern (Hernández-Gras, Francesc, *et al.* 2015) Lipocalins are believed to interact with the external face of the membrane through a hydrophobic loop. Hydropathy profiles of *Arabidopsis* TIL proteins shows that this loop extends over a short stretch of over eight hydrophobic residues (Hernández-Gras, Francesc, *et al.* 2015).

Plants synthesize a variety of steroids that function as signaling molecules (Wang, Zhi-Yong, et al., 2006). Brassinosteroids (BR) are known to regulate multiple aspects of physiological responses, such as male fertility (pollen development), senescence, cell elongation and division (Clouse, Steven D, 2011). It also has a role in temperature response. Treatment with 24-epibrassinolide (Charron, Jean-Benoit Frenette, *et al.*, 2002) increases heat and cold tolerance within plants. This enhanced resistance is attributed to membrane stability, and suggests that part of temperature response in plants involve brassinosteroids as signaling molecules. This study investigate if pretreatment of gametophytes with 24-epibrassinolide can increase tolerance to cold stress. Moreover, this study investigates if 24-epibrassinolide affect the expression of a putative TIL gene in *Ceratopteris richardii*.

METHODOLOGY

A. Physiological characterization of gametophytes of *Ceratopteris richardii*

Liquid C-Fern® media (Cat. #156782) and C-Fern® agar (Cat. #156781) were purchased from Carolina Biological and prepared according to manufacturer's instructions. Pre-sterilized dry spores of *Ceratopteris richardii* were purchased from Carolina Biological (Catalogue #156728). Prior to plating, spores were imbibed with sterile distilled water in the dark for 7 days to allow for synchronization. Spores were plated on the C-Fern® agar and were allowed to develop under continuous white light. Distilled water was used as the liquid media. Germination was measured by the emergence of rhizoids through the spore-coat. After six days of developing, the concentrations of 24-epibrassinolide (Sigma Aldrich Cat. #E1641) was measured - 10 µM - and applied to two of the agar plates. Two control groups were also chosen for reference. Percent germination and relative rhizoid length was taken into account and measured starting on the tenth day using a compound light microscope. On the sixteenth day, both plates for control and 24-epibrassinolide (EBL) were flooded with 2 mL more of respective treatments (control flooded with distilled water). On the seventeenth day, one agar plate treated with EBL and one control plate was treated with cold stress (4-10 ^{o}C) for 24 hours. On the eighteenth day measurements of percent germination were also taken, and the plates were returned to normal conditions. Through analysis with a stereo light microscope, inflation of gametophytes was observed exclusively in gametophytes treated with cold stress. Images of room temperature control and EBL were taken, as well as cold stress control and EBL. The percent of gametophytes with inflations were measured and the number of inflations per gametophyte was also measured.

B. Bioinformatics analysis

To identify an orthologue of the TIL gene in *Ceratopteris*, an Expressed Sequence Tag cDNA library of *Ceratopteris richardii* was screened by BLAST. The sequence was then characterized using Open Reading Frame Finder (ORF) on the National Center of Biotechnology Information (NCBI) database.

C. Domain Analysis

Multiple sequence alignments of the full-length deduced amino-acid sequences of the TIL gene were performed by Clustal Omega (www.clustal.org/omega). Localization and modifications on the domain were discovered through a journal (Frenette Charron, Jean-Benoit, *et al.*, 2002) for essential motifs and structurally conserved regions (SCRs).

D. Phylogenetic characterization of TIL homologues

Protein sequences of TIL genes were obtained from the NCBI database and phytozome. Multiple sequence alignments were performed using the BioEDIT platform and indels (insertions/deletions) were removed. Formatted sequences were then used to construct unrooted phylogenetic trees in the neighbor-joining method formatting using the MEGA 6.0 software.

E. RT-PCR

1 µg of RNA was reverse transcribed using the Invitrogen SuperScript® III Platinum® One-Step RT-PCR Kit (Catalogue #A11732-020). The primers used: CrTIL-forward: CGC CAT TAC TTT TAT and Cr-TIL-reverse: GTT GTG CAG TCA TGG TG. PCR cycles were performed using the following conditions: denaturation at 94° C for 30 seconds, annealing at 55° C for 30 seconds, and extension at 72° C for 1 minute; final extension was performed at 72° C

for 3 minutes. Amplification of the PCR products was analyzed using agarose gel electrophoresis. DNA was visualized using 1% agarose gel in 1xTBE solution (89mM Boric Acid, 89mM Tris Base, 2mM EDTA) with SYBR Safe DNA stain and UV light. Intensity of DNA was analyzed using ImageJ. Number of cycles ranged from 25-35.

RESULTS

There have been no studies that characterized the inflation of gametophytes in ferns. Thus, data was taken on the gametophytes - under the same temperature conditions - to elucidate a connection between cold and EBL on inflation. As seen in Figure 4, inflations were exclusive for gametophytes exposed to cold stress. Comparison between untreated cold stress and EBL cold stress showed that gametophytes treated with EBL exhibited significantly less inflation. Furthermore, gametophytes treated with EBL also exhibited significantly less inflations per gametophyte relative to the control.

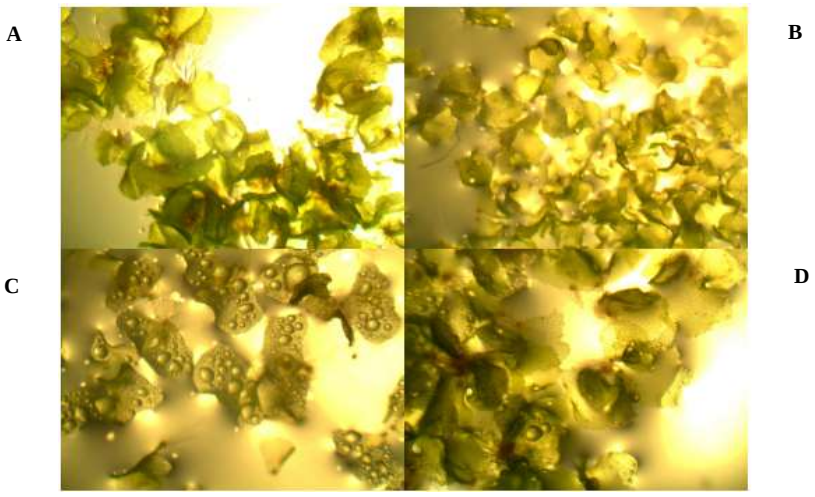


Figure 1. **The effect of temperature stress on development of gametophytes.** (A)Room temperature (22-25 °C) control, (B) room temperature and 24-epibrassinolide, (C) cold stress (4-10 °C), (D) cold stress and 24-epibrassinolide.

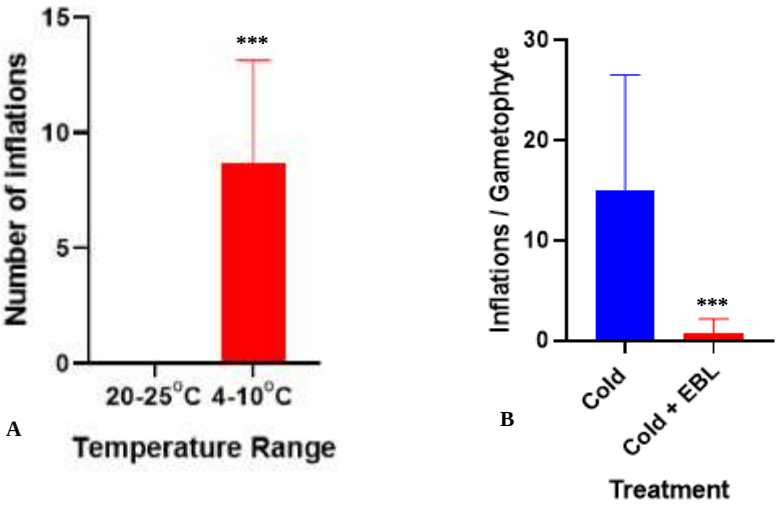


Figure 2: **Effect of cold stress (4-10°C) and 24-epibrassinolide treatment on number of inflations and inflation/gametophyte during gametophyte development of Ceratopteris richardii**. (A) Number of inflations for room temperature and cold stress. (B) Inflations/gametophyte for cold control and cold 24-epibrassinolide. Significance was determined with respect to the control using by a one way ANOVA (Dunnet's Multiple Comparison Test). $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.0001$ = ***

It is known that cold and freezing stress lead to a decrease in the fertility of plants. However, there have been no studies that have shown the correlation between fertility and inflation of gametophytes. Hence, the sporophyte count was taken. Gametophytes exposed to room temperature exhibited significantly greater fertility relative to those exposed to cold stress. Furthermore, gametophyes treated with 24-epibrassinolide under cold stress exhibited significantly greater fertility relative to cold stress, thus correlating the brassinosteroid pathway with cold stress response.

Figure 1. **The effect of temperature stress on sporophyte development** (A)Room temperature (22-25 °C) control, (B) room temperature and 24-epibrassinolide, (C) cold stress (4-10 °C), (D) cold stress and 24-epibrassinolide.

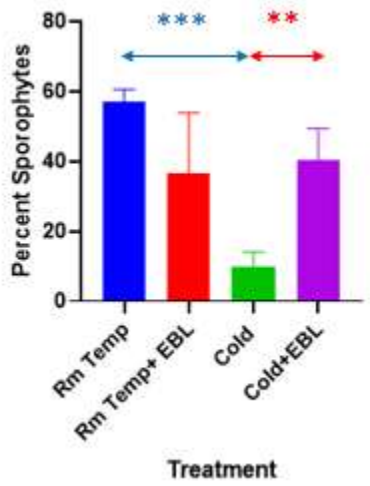


Figure 2: **Effect of cold stress (4-10°C) and 24-epibrassinolide treatment on sporophyte count of *Ceratopteris richardii***. Significance was determined with respect to the control using by a one way ANOVA (Dunnet's Multiple Comparison Test). $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.0001$ = ***

To determine the effect of cold stress on cell division and cell elongation of cell of the prothalli, 17-day-old gametophytes were treated for 24 hours under conditions of low temperature (4-10 °C) in continuous white light. Gametophytes were then transferred to room temperature for an addition 24 hours of growth. Images of prothalli were taken under a compound light microscope. Area of the prothalli were measured by Image J and the number of cells were manually counted.

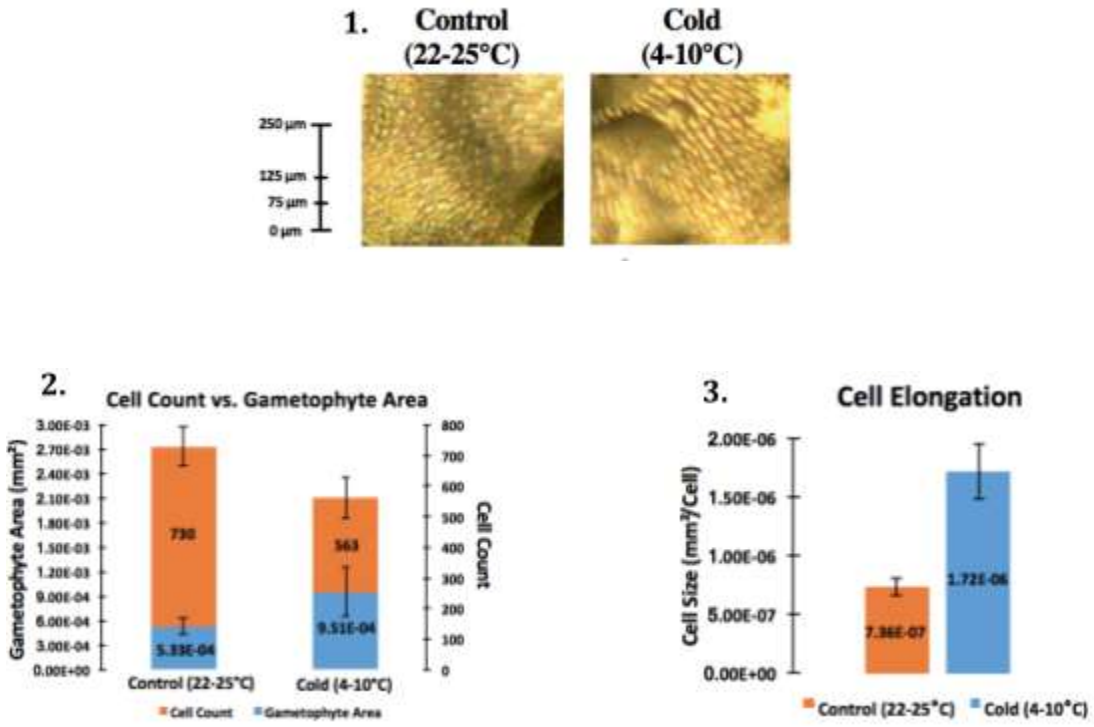


Figure 3: **Effect of cold stress (4-10 °C) on the average cell area and number of cell per gametophytes.** Graphs indicate cell count, gametophyte area and cell elongation. Two-tail distribution T-test of cell count, gametophyte area, and cell elongation data yielded p-values of 2.645×10^{-05}, 2.12601×10^{-13}, and 1.75×10^{-11} respectively.

As seen in Figure 3, exposure to cold temperature reduces the area of the prothalli. Cellular examination revealed that cold stress reduced cell division but promoted cell elongation.

To determine the molecular effect of temperature on gametophyte development, the role a putative TIL gene was characterized in *Ceratopteris richardii*. To identify an orthologue of a Temperature-Induced Lipocalin (TIL) gene in *Ceratopteris*, known TIL genes from other plants (e.g. *Arabidopsis thaliana*) was used as a probe to identify genes of similar sequences in *Ceratopteris*. An Expressed Sequence Tag cDNA was prepared (by mentor) from light-grown gametophyte tissue. Using Basic Local Alignment Search Tool (BLAST), a putative gene was identified in *Ceratopteris richardii* (Figure 4).

Figure 4: Putative TIL gene in *Ceratopteris richardii*.

Analysis of the gene sequence identified a continuous open reading frame of 198 amino acid residues (Figure 4).

Species	% Identity	E-value	Accession #
Arabidopsis thaliana	59.12%	8e-80	NP_200615.1
Glycine max	59.34%	2e-79	NP_001235845.2
Triticum aestivum	57.92%	2e-81	ABB02379.1
Aegilops tauschii	56.83%	9e-80	XP_020171391.1
Phoenix dactylifera	57.14%	1e-79	XP_008794329.1
Hordeum vulgare	56.28%	2e-79	ABB02380.1
Solanum tuberosum	65.85%	4e-62	PGSC0003DMP400013911
Theobroma cacao	58.15%	5e-86	Thecc1EG037092
Salix purpurea	60%	2e-84	SapurV1A.0347s0210.1.p
Ananas comosus	55.85%	8e-81	Aco016911.1
Oryza sativa	56.35%	6e-81	LOC_Os08g34150.1
Sorghum bicolor	55.49%	8e-77	Sobic.004G213100.1.p

Table I. Similarity of CrTIL polypeptide with known TIL proteins. Analysis was performed using BLAST comparing polypeptide sequences.

Using the predicted protein sequence as a query, BLAST analysis was performed using the NCBI database. A BLAST analysis compares the sequence at hand with every other sequences in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). Comparison of the polypeptide sequences revealed that the putative TIL gene from *Ceratopteris* (designated CrTIL) has high percent identities (~50%) to known TIL protein sequences (Table 1). In addition to high percent identity, there are low E-values, indicating that similarity is statistically significant.

Protein sequences of TIL genes were acquired from the NCBI database and the Phytozome database. Multiple sequence alignments were performed using the BioEDIT

platform, and indels (insertions or deletions) were removed. Formatted sequences were then used to construct phylogenetic trees using the Neighbor-Joining Method (using MEGA6 software).

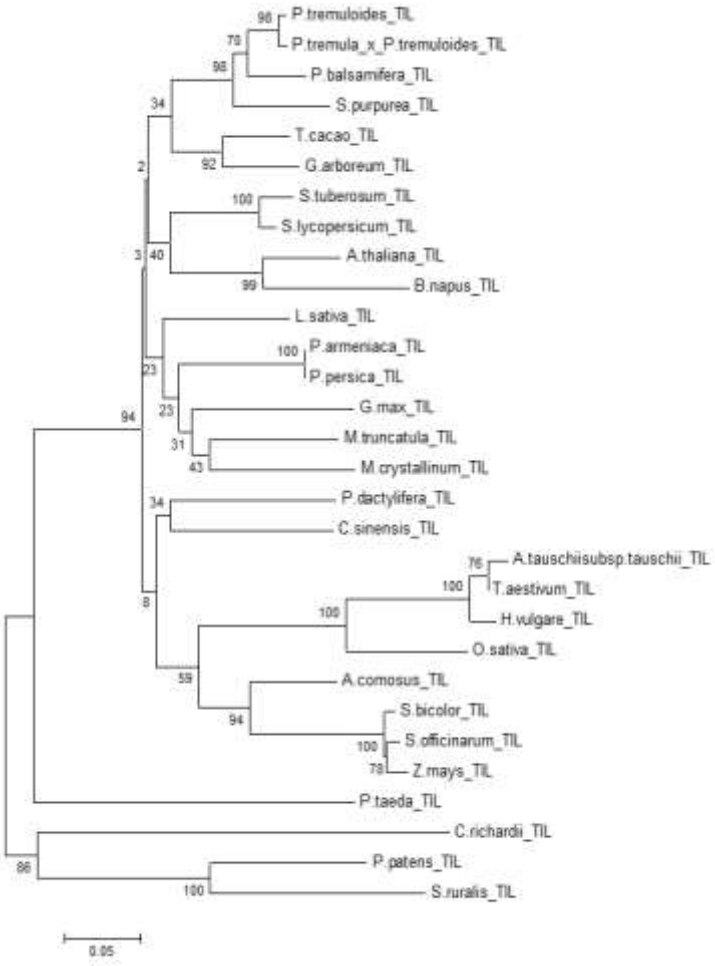


Figure 5: **Phylogenetic tree of the TIL sequence in *C. richardii* and known orthologues**. Neighbor-joining method was used to construct the tree.

Analysis of the phylogenetic tree shows that there is a distinct clade of TIL genes from seedless plants and seed plants. CrTIL gene is most closely related to the TIL genes in *Physcomitrella* (a moss) and *Selaginella* (a lycophyte); both of these plants are also seedless plants. A division between the angiosperms was observed, and it was inferred that this was

because of the speciation between the TIL genes of monocots (one cotyledon) and dicots (two cotyledons).

To determine if CrTIL contain all of the essential motifs of a TIL protein, a domain analysis was performed.

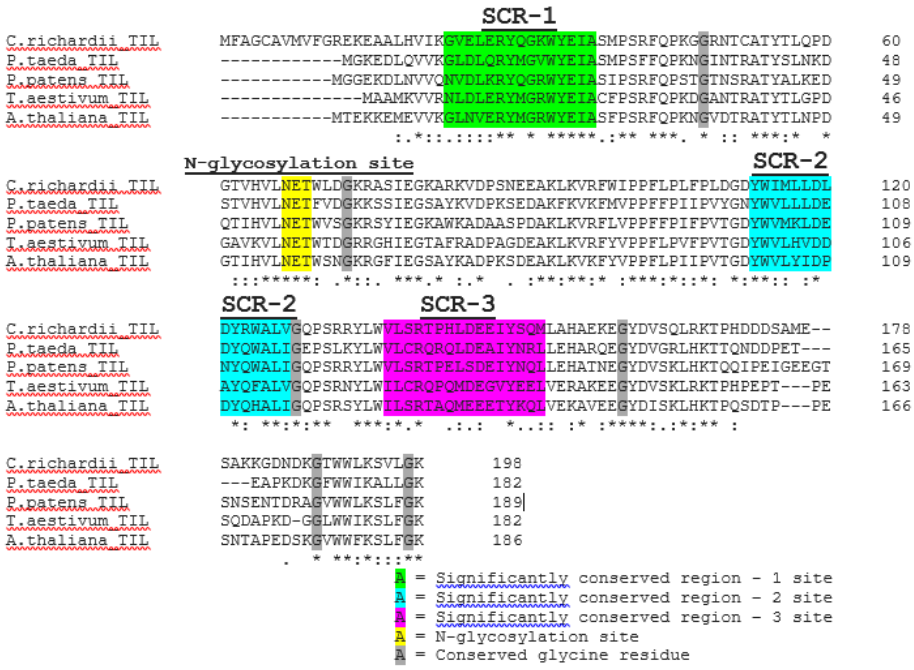


Figure 6. **Domain analysis of the TIL protein sequences.**

Multiple sequence alignments of the full-length deduced amino acid sequences of TIL genes were performed using the Clustal Omega software. Localization and modifications on the domain were identified using other known TIL genes as reference (Charron *et. al*, 2002). As seen in Figure 6, the sequence identified from the cDNA library in Ceratopteris codes for a full-length polypeptide. In the TIL sequence, there is generally a putative N-glycosylation site as well as the aforementioned Significant Conserved Regions (SCRs) (Charron *et al.*, 2002). Using the sequence alignment from Clustal Omega, it was found that all three of the SCRs were conserved

in *C. richardii*, as well as the N-glycosylation site. Furthermore, various glycine residues were conserved.

The species used in the domain analysis ranged from conifers, monocots, dicots, ferns and mosses. Thus, with all the sites and motifs conserved in the TIL sequences of the organisms, mutations in the sequence of TIL is most likely selected against.

The role of CrTIL as a membrane-associated protein was further elucidated through the use of hydropathy plots. Hydropathy profiles of *Arabidopsis* TIL proteins shows that this loop extends over a short stretch of over eight hydrophobic residues (Hernández-Gras, Francesc, *et al.*, 2015). Through simultaneous analysis of the protein sequence and HPR motif of the hydropathy plot, it was found that all TIL orthologues - including the putative CrTIL - contained the PPFLPIIP sequence.

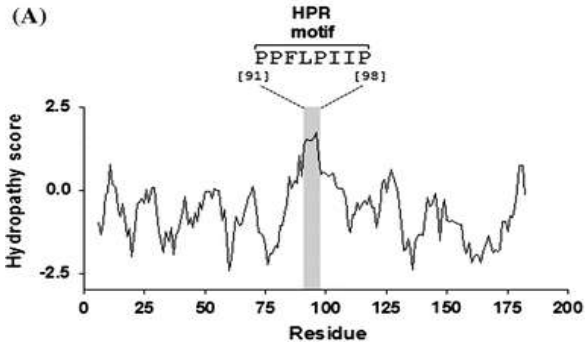


Figure 7: **Hydropathy plot containing the HPR residue.** The gray highlighted section shows the location of the hydrophobic proline-rich sequence responsible for forming the loop.

To analyze the expression of CrTIL in *Ceratopteris richardii* under cold stress, semi-quantitative RT-PCR was performed. Seventeen-day-old gametophytes were exposed to 24 hours of cold (4-10^{o}C). Total RNA was isolated from gametophytes, reversed transcribed into cDNA and amplified using the primers for CrTIL and CriActin. The beta actin gene was used as control and to standardize the samples (Figure 8). As seen from RT-PCR results, there is a significant

increase in the expression of TIL after exposure to cold stress. However, there is significantly more expression of TIL when it is exposed to EBL under room temperature (Figure 8).

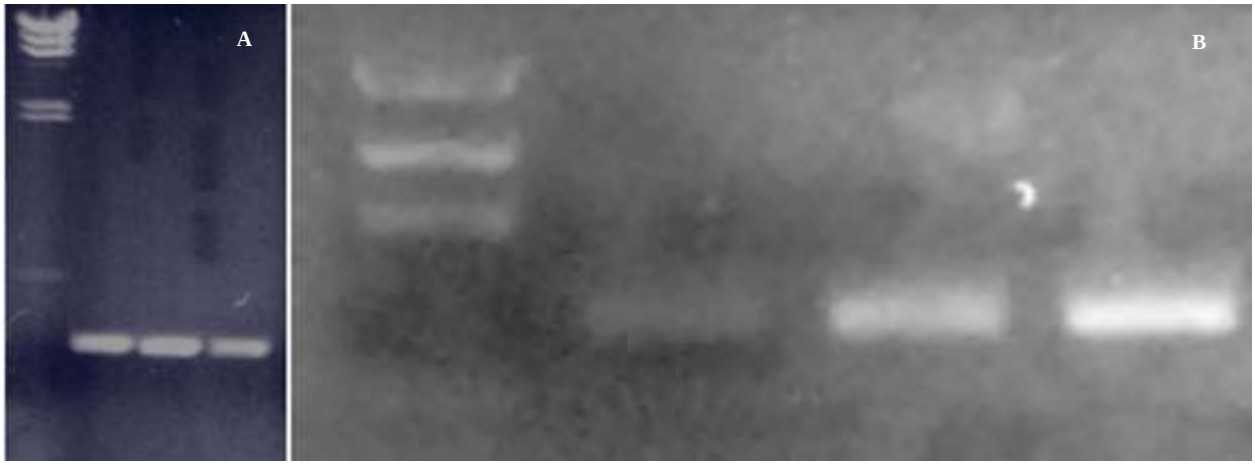


Figure 8. **CrTIL gene expression under cold stress and EBL measured using RT-PCR**. Actin housekeeping gene used as internal control. Expression for gene exposed to cold stress and treated with 24-epibrassinolide was compared with room temperature untreated. Samples were treated at 4-10 °C during the cold stress stage and 22-25 °C during room temperature stage. 24-epibrassinolide-treated sample was treated with 10µM

DISCUSSION

Lipocalins are a functionally diverse family of genes that have been known to play important roles in various physiological functions of plants, such as modulation of cell growth and metabolism, binding of cell-surface receptors, nerve growth and regeneration, regulation of the immune response, smell reception, cryptic coloration, membrane biogenesis and repair, induction of apoptosis, animal behavior, and environmental stress response (Akerstrom et al., 2000; Bishop, 2000; Frenette Charron et al., 2002). Temperature induced lipocalin (TIL) is a subsection of this family, and it is known to induce thermotolerance in plants. For example, a study indicated that low temperature treatment on *Triticum aestivum* induced two TIL isoforms (Charron, Jean-Benoit Frenette, et al., 2005). It was shown that heat-shock induced TIL-1 in *T.*

aestivum, but not TIL-2 (Charron, Jean-Benoit Frenette, et al., 2005). Furthermore, overexpression of TIL in *Arabidopsis thaliana* led to increased survival rates at freezing temperature (Tominaga et al., 2006; Uemura et al., 2006). Analysis of the RT-PCR data indicates that, relative to the control, the CrTIL gene is upregulated during cold stress. This suggests that CrTIL plays a role in cold stress adaptation. CrTIL was shown to have this hydrophobic loop through domain analysis. It contained the essential motif of the hydrophobic loop (Hernández-Gras, Francesc, *et al.*, 2015). Furthermore, CrTIL contained the three structurally conserved regions (SCR), which further corroborates CrTIL as a temperature lipocalin.

In a study conducted by Charron, Jean-Benoit Frenette et al., localization of TIL in *Arabidopsis thaliana* was determined through transient expression analysis of green fluorescent protein (GFP) fusion proteins in onion (*Allium cepa*) epidermal cells to establish the subcellular location of the *At*TIL-1 protein (Charron, Jean-Benoit Frenette, et al., 2005). The results show that the GFP::TIL fusion accumulates specifically at the plasma membrane, whereas the fluorescence is visible throughout the cell when the GFP protein is in its native state (Charron, Jean-Benoit Frenette, et al., 2005). In the same study, biochemical fractionation analysis was also performed to determine the localization of TIL in *Triticum aestivum*. Wheat protein extracts were prepared and subjected to SDS-PAGE and western-blot analyses (Charron, Jean-Benoit Frenette, et al., 2005). Immunoblot results indicate a similarly high accumulation of *Ta*TIL-1 in an enriched PM fraction of cold-acclimated wheat but not in nuclei (Charron, Jean-Benoit Frenette, et al., 2005). Hence, plasma membrane localization seems to be conserved among temperature induced lipocalins. The lipocalin fold, due to its hydrophobic nature, allows for functionality in the plasma membrane. CrTIL was shown to contain the hydrophobic proline-rich (HPR) residue

that is conserved among lipocalins. The HPR residue characterizes lipocalins' functionality in the plasma membrane.

In *Arabidopsis thaliana*, overexpression of TIL1 led to increased survival rates at freezing temperature (Tominaga et al., 2006; Uemura et al., 2006). However, in another study, overexpression of TIL in *Arabidopsis* suggested that, while the induction of TIL1 by acclimation heat treatment is associated with acquired thermotolerance, over-expression of TIL1 by tenfold did not significantly enhance **basal tolerance**, suggesting that TIL1 is essential but not sufficient for tolerance against **heat stress** (Chi, Wen-Tzu, et al., 2009). Therefore, while there is variance in the types of stress that TIL is selectively tolerant to among different species, TIL is nevertheless selectively tolerant in regards to the stress it is exposed to. It is likely that CrTIL is selectively tolerant as well.

While TIL is known to play an important role in cold stress response in plants, the molecular function of TIL is yet to be elucidated. In plants, non-enzymatic lipid peroxidation produces structurally similar products known as phytoprostanes (Chi, Wen-Tzu, et al., 2009). In this process, the initial phytoprostane formed is phytoprostane G1 (PPG1). PPG1 is highly unstable and spontaneously decays into malondialdehyde(MDA) and other alkanes and alkenes (Sattler *et al.* 2006). PPG1 is also the precursor of at least six different classes of phytoprostanes (Mueller 2004). MDA and some of the phytoprostanes, which are electrophiles, can modify proteins as well as other cellular components and cause deleterious effects on cells (Sattler *et al.* 2006). It is possible that TIL1 binds and scavenges these reactive phytoprostane species as its animal homologs do. In this study, the growth of mutant plants was more sensitive to *tert*-butyl hydroxide treatment than that of wild-type plants, which has been shown to dramatically increase the level of several phytoprostane species (Thoma *et al.* 2003). Results of

the RT-PCR indicate that there may be a correlation between the brassinosteroid pathway and CrTIL. This was because CrTIL was upregulated the most when it was exposed to 24-epibrassinolide. Relative to CrTIL exposed to cold stress and room temperature, it was expressed more significantly.

Heat stress and cold stress have been shown to be closely related to production of Reactive Oxygen Species (ROS). Heat stress caused impairments in mitochondrial functions and result in the induction of oxidative damage that manifested in lipid peroxidation (Suzuku et al., 2005). The transcript and protein level of many ROS-scavenging enzymes was also found to be elevated under heat stress (Suzuki et al., 2005). In a study (Larkindale et al., 2002) demonstrated that protection against heat-stress induced oxidative damage involves calcium, abscisic acid (ABA), ethylene, salicylic acid (SA). Mutants deficient in these substances showed defects in acquired heat tolerance (Suzuki et al. 2005). Cold stress was shown to enhance the transcript, protein, and activity of different ROS-scavenging enzymes (Suzuki et al. 2005). It was also shown to induce hydrogen peroxide accumulation in cells (O'Kane et al. 1996). *Arabidopsis* frostbite1 (*fro1*) mutant displayed reduced expression of cold-responsive genes such as RD29A, KIN1 COR15A, and COR47, and accumulated ROS constitutively (Lee et al., 2002). TIL was implicated to have a possible role in the regulation of ROS species. Suppression of TIL1 in *Arabidopsis* let to higher sensitivity to oxidative stress and accumulation of higher levels of lipid peroxidation product, MDA, after heat stress (Chi et al. 2009). Furthermore, AtTIL knock-out plants are more sensitive to sudden drops in temperature, and they were also shown to accumulate significantly higher levels of ROS species (Charron *et al.,* 2008). On the other hand, overexpression of AtTIL was shown to increase tolerance to stress caused by freezing and light (Charron et al. 2008). Thus, it is possible that TIL plays an essential role in the regulation of

ROS species when the organism is placed under severe temperature stress, and it was also shown to alleviate the accumulation of ROS species in light stress as well. Nevertheless, the mechanism in which the TIL gene accomplishes this is unknown.

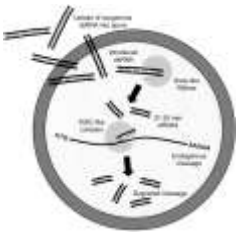
CONCLUSION

When gametophytes of Ceratopteris was exposed to cold stress, there was a reduction in cell division and prothalli area, but an increase cell elongation. An inflation of the gametophytes was also observed which reduced subsequent development of gametophytes. Treatment of gametophytes with 24-epibrassinolide can reduce this inflation and promote development of gametophytes are subsequent cold stress.

An orthologue of a Temperature Induced Lipocalin (CrTIL) was identified in Ceratopteris. There is an increase expression of CrTIL when plants are treated with 24-epibrassinolide or exposed to cold stress, suggesting a possible role in regulating development.

FUTURE STUDIES

Future studies will include knockdown of the expression of CrTIL through the technique, RNA interference. Double stranded RNA will be used to lower the expression of CrTIL. Gametophytes will then be exposed to cold stress and the response will be measured.



(Stout et al; 2003)

One of the main effects of BR signal transduction is the inactivation of Brassinosteroid Insensitive 2 (BRI2) kinase, a downstream negative regulator of BR signaling. Inactivation of BRI2 leads to the activation of two transcription factors: Brassinazole-Resistant 1 (BZR1) and BR1 EMS suppressor 1 (BES1). BZR1 and BES1 regulate the expression of hundreds of genes responsible for physiological responses such as anther and pollen development (Clouse, Steven D, 2011). Future studies would identify orthologues of these genes and determine if there expression is also affected by cold stress.

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