Elevated temperature and DNA methylation Affects Mago nashi expression and sexual development in *Ceratopteris richardii*

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ABSTRACT

Climate change, specifically elevated temperature, has been previously correlated with a significant decrease in crop yield. High temperatures have been shown to increase male sterility, reduce pollen viability and decrease seed yield. The molecular mechanisms of how temperature stress affects sexual differentiation and fertility is not well characterized. This study used the fern *Ceratopteris richardii* as a model organism to understand how elevated temperature would affect sexual development of gametophytes and subsequent sporophyte production.

To identify possible genes that might be regulated by high temperature and are required for sexual development, we analyzed microarray data from the seed plant *Arabidopsis thaliana*. In the microarray data, the gene Mago nashi was significantly upregulated during elevated temperature. Using BLAST, we identified an orthologue of Mago nashi in *Ceratopteris*. Phylogenetic analysis revealed that Mago nashi evolved before the separation of seed from seedless plants and the structural domains are highly conserved, suggesting key evolutionary importance. RT-PCR revealed that there is greater expression of Mago nashi in males than hermaphrodites. Factors that promoted male development in *Ceratopteris* such as high temperature (28^{o}C vs 24^{o}C) and 5-azacytidine, an inhibitor of DNA methylation, resulted in greater expression of Mago nashi when compared to the controls.

This study demonstrated that an orthologue of the Mago nashi gene is affected by temperature changes. Regulation of the gene expression also involved an epigenetic mechanism i.e. DNA methylation. Future knockdown studies of Mago nashi via RNAi would further support the importance of Mago nashi in sexual differentiation and fertility.

INTRODUCTION

Climate change, more specifically elevated temperature, has been previously correlated with a significant decrease in crop yield. Elevated temperatures has also been shown to reduce pollen production and fertility in various plants such as *Phaseolus vulgaris* (Gross and Kigel, 1994), *Brassica napus* (Young *et. al*, 2004), and *Lycopersicon esculentum* (Abdul-Baki and Stommel, 1995). With the world population predicted to grow to about 9 billion in 2050, it is crucial that a stable food supply is established. The United Nations Food and Agriculture Organization predicts that it is necessary that crop yield increase by 70% in order to support the estimated population in 2050 (Hofstrand, 2014).

The United States of America is the world's largest agricultural producer and plays a significant role in global food production. Global warming has been seen to be beneficial for crop prediction, however after temperatures surpass 28℃, the production may dramatically decreases. For example, corn production declines at 29℃, soybeans at 30℃ and cotton at 32℃ (Schlenker and Roberts, 2009).

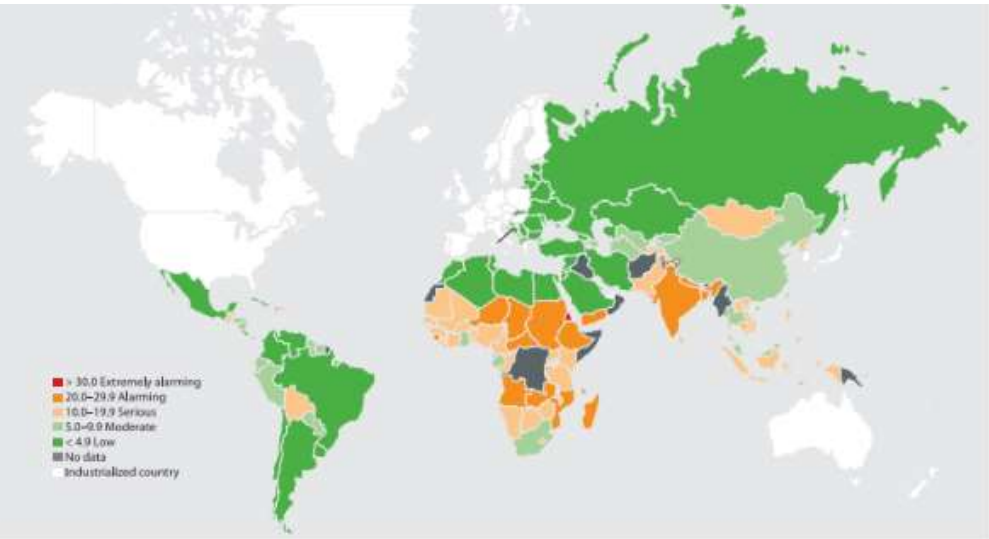

Figure 1: The Global Distribution of Hunger, which is quantified by the 2012 Global Hunger Index.http://denning.atmos.colostate.edu/readings/Impacts/FoodSecurity.Science-2013-Wheeler-508-13.pdf

The estimated decrease in the production of maize is expected to be 4.2 million tons in 2025 and 11.6 million tons per year in 2055. The dramatic decline in crop production may become a problem throughout the world, especially in Africa, as seen in the diagram above.

It is also estimated that in the US, for every 1°C increase in the average temperature, there will be a 1.2% loss in profit from every industry in the US combined (Hsiang, *et al*, 2017). This can have major implications on the economy of the US because if temperatures continue to rise, the 1.2% cost will add up and eventually have a major impact on the economy. Even further, it is expected that the bottom third of US counties will experience a 2-20% decrease in income by the end of the 21st century (Hsiang, *et al*, 2017). Climate change has the ability to play a significant role in the economy and if temperatures continually rise, global economies will be hurt and general livelihood will be reduced

Elevated temperature is known to reduce crop yield, where increases in temperature are detrimental to pollen development. This is because pollen production and development is heat sensitive and highly regulated. Specifically, both the microsporogenesis and microgametogenesis phases are extremely sensitive to heat (Mueller and Rieu, 2016). However, the mechanism of heat sensitivity is not well understood.

Mago nashi has been shown to play a key part in the growth and development of *Drosophila* embryos (Swidzinski *et. al*). It has also been shown that in *Drosophila,* Mago nashi and Y-14 are responsible for eye development. In *C. elegans* Mago nashi is also involved in sexual differentiation (Swidzinski *et. al*). In *Mus musculus*, Mago nashi is related to cell cycle regulation. In animals, Mago nashi is known to interact with *oskar* and *gurken* mRNA. *Gurken* mRNA is known to be responsible for the first reciprocal inducing event in the oocyte. The *gurken* mRNA is crucial to egg chamber development and is necessary when defining the

anterior-posterior (AP) and the dorsal-ventral (DV) axes in the oocyte, this occurs during oogenesis (Mohr, *et. al*, 2001). *Oskar* mRNA has been shown to be necessary for the development of posterior lobe of embryos as well as non-viable eggs (Gong, *et. al*, 2014).

Mago nashi is a highly conserved RNA-binding protein that is involved in germ cell differentiation. It is a ubiquitous gene found in both animals and plants. Mago nashi has been shown to be highly conserved throughout numerous species of plants and animals (Swidzinski *et. al*). It is known that Mago nashi binds to the pre-messenger RNA for OsUDT1, or undeveloped tapetum 1 which is a transcription factor in rice (*Oryza sativa*). OsUDT1 is required for fertility in *Oryza* and plays a significant role in tapetum development (Jung, *et. al*, 2005).

Mago nashi has been found in a range of species of plants such as *Oryza sativa*, *Physalis floridana*, *Marsilea vestita*, and *Arabidopsis thaliana* (Swidzinski *et al*., 2001; He *et al*., 2007; Weele *et al*, 2007). Not surprisingly, Mago nashi in plants also affect the sex determination or the early development in plants, just as it does in animals. In *Marsilea*, Mago nashi was found to affect spermatogenesis; in *Physalis*, the absence of Mago nashi promoted male fertility (He *et al*., 2007; Weele *et al*, 2007). Similar to *Caenorhabditis*, the absence of Mago nashi in *Arabidopsis* led to non-viable seeds (Park *et al*., 2009). However, examination of Mago nashi in higher plants is very difficult due to the complexity of pollen and seeds (Bushart *et al*., 2007). The molecular mechanisms such as, the mRNA that Mago nashi binds to is not well understood in plants. To understand the mechanisms of gametophyte development in higher plants, it is important to study these mechanisms in plants such as ferns (Banks, 1999).

Mago nashi has been shown to play a role in export of mRNA from the nucleus to the cytoplasm in eukaryotic organisms. Mago nashi has co-evolved with another mRNA binding protein, Y-14 (Gong, *et. al*, 2014). Y-14 and Mago nashi are both involved in export of mRNA

into the cytoplasm along with export factors barentz (Btz) and eukaryotic initiation factor 4a-III (eIF4AIII) (Roignant and Treisman, 2010). These export factors, along with Mago nashi and Y-14 also play a key role in the function of the Exon Junction Complex or EJC and development in *Physalis floridana* (Gong, *et al*, 2018). It was shown that the knockdown of these key EJC components such as Mago nashi and Y14 in *Oryza sativa* resulted in much shorter plants compared to the wild type (Gong and He, 2014). This suggests the momentous role that these components of the EJC play in the early development of plants.

One such plant that has arisen as a model fern is the aquatic fern, *Ceratopteris richardii* (Cooke *et al*., 1995; Chasan, 1992). The gametophytes of *Ceratopteris* are either characterized as male or hermaphrodites. Since ferns have both characteristics of higher plant system (sporophyte) and a lower plant system (gametophyte), examination of *Ceratopteris* is highly advantageous because growth and development can be examined separately in each different developmental stage (Hickok *et al*., 1995; Banks, 1999). The sex expression of gametophytes in *Ceratopteris* is determined by a chemical messenger, antheridiogen. Antheridiogen promotes the male sex organ, antheridia (Warne and Hicock, 1988). The hermaphrodite produce two types of sex organs, the archegonia; which contains the egg and the antheridia; which contains the sperm. Two of the mutants that were characterized in *Ceratopteris* are the *him1* and her1 (Banks, 1994; Banks, 1999). *him1* and *her1* display abnormalities in sexual development (Spiro et al.,2008; Banks 1994). *him1* favors the development of male gametophytes, while *her1* favors the development of hermaphroditic mutant.

When the gametophytes are exposed to water, the mature antheridia release sperm. The sperm is then attracted to archegonia which release a chemical signal. This allows the sperm to locate as well as fertilize the eggs. The fertilization of the egg leads to the formation of the

diploid stage of the cell. The fertilized egg then further develops into the sporophyte stage of development (Bent Tree Press).

The role of Mago nashi in *Ceratopteris richardii* has not been identified. This study examines the role of Mago nashi in sexual differentiation of the male and hermaphrodite in response to temperature change.

METHODOLOGY

Heat Stress

Dark imbibed spores were plated on C-fern Media (Carolina Biological) and allowed to develop for 7 days. All experiments were performed in triplicates. Germinating spores were allowed to developed either 22-24°C (normal growth condition) or 26-28°C (elevated temperature).

Inhibition of DNA Methylation

Spores were incubated in the dark for 7 days with the 25 µM 5-Azacytidine, 5-AC (Sigma Aldrich). Since this chemical was highly toxic, it was prepared and handled by mentor. Personal protective devices includes goggles, gloves, lab coats and fume hood. After 7 days of incubation, spores were plated onto C-fern media (Carolina Biological) and allowed to develop under continuous white light. The ratio of males to hermaphrodite was assessed at 10 days. The number of sporophytes produced was measured after 30 days.

Bioinformatics Analysis

Using the Basic Local Alignment Search Tool (BLAST), we screened an Expressed Sequence Tag (EST) cDNA library of Mago nashi to find a possible orthologue of it in *C. richardii*. Sequence was analyzed using Open Reading Frame (ORF) finder. Phylogenetic analysis was performed using MEGA 7 software. We used Clustal Omega to create a sequence

alignment using the Mago nashi orthologues in selected organisms. Key motifs, binding sites, and structural formations were identified and mapped onto the sequence alignment chart and the conserved regions across the selected organisms and *C. richardii* were identified.

RT-PCR

Purified RNA (by mentor) was obtained from developing gametophytes. RNA was reversed transcribed into cDNA using a cDNA kit from Invitrogen. In a sterile RNase free eppendorf tube, 1 µl of *Oligo dT primer* (10 µM), 9 µl of *RNA* (1µg) and 2 µl of 10 nM *dNTP Mix* was added. This reaction was incubated at 65°C for 5 minutes to denature the RNA and was placed on ice. Then, the 5X *cDNA Synthesis Buffer* was vortexed for 5 seconds immediately before use. The reaction mix of 4 µl of 5x *cDNA Synthesis Buffer*, 1 µl of 0.1M *DTT*, 1 µl of *RNase OUT* (40U\µl) and 1 µl of *ThermoScript* (15units/µl) was prepared on ice. This reaction mix was mixed gently and 8 µl was pipetted into the reaction tube with the primer and RNA. This sample was placed in a preheated thermal cycler. The reaction was incubated for 60 minutes at 50°C. The reaction was incubated at 85°C for 5 minutes to stop the reaction. 1 µl of RNase was added and incubated at 37°C for 20 minutes. The first strand cDNA was used immediately for a PCR reaction. To amplify the cDNA, the following primers were used: CrMN-F: AAC AAC TGG CCA GAG CCT GAC, CrMN-R: AAG ATG CGC AAT CCC TCT GGA. Thermal cycling was performed with the common amplification parameters of one cycle of initial denaturation at 94°C for 3 minutes, 30-35 cycles of denaturation at 94°C for 30 seconds, 30-35 cycles of annealing at 55°C for 30 seconds, 30-35 cycles of extension at 72°C for 1 minute, 1 cycle of final extension at 72°C for 10 minutes.

RESULTS

Preliminary Data:

High temperature has been shown to decrease fertility and pollen production in seed plants. Preliminary analysis of microarray data (Figure 1) in *Arabidopsis thaliana* indicated that elevated temperature and 5-Azacytidine, an inhibitor of DNA Methylation, affects the expression of Mago nashi.

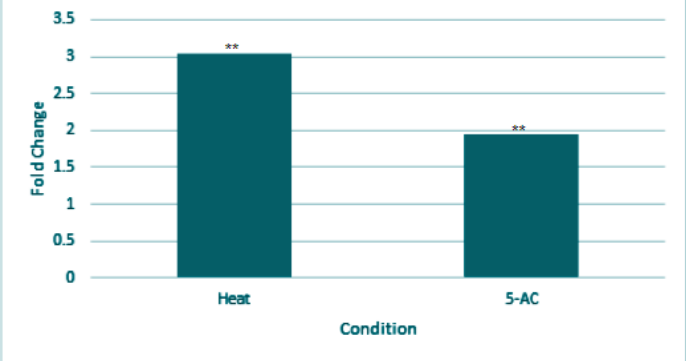

Figure 1: Factors that affect the fold change in Mago nashi under heat and 5-AC in *Arabidopsis thaliana*. Asterisks represent statistical significance, two asterisks indicate a p value < .01.

As seen in Figure 2, when germinating spores are subjected to heat stress (28°C), there is significantly more males that developed than hermaphrodites.

Similarly, if the germinating spores were treated with 5-Azacytidine (5-AC), there is also significant amount of males produced. For each conditions, hundreds of gametophytes were

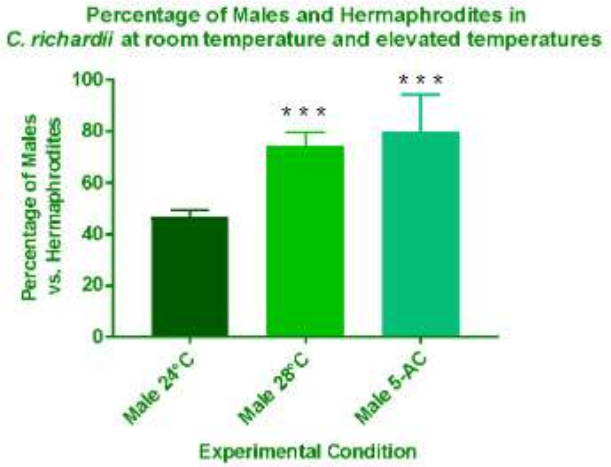

Figure 2: Percentage of males out of 100 in *Ceratopteris richardii* under various conditions (24°C, 28°C, no treatment, and 25μM 5-AC). Error bars represent standard deviation. Asterisks represent statistical significance and p values < .001.

counted and a student T-test was performed. All experiments were performed in triplicates to ensure accuracy and reliability.

To determine if high temperature or 5-AC affected fertilization, the number of sporophytes were measured. As seen in Figure 3, there was a decreased percentage of

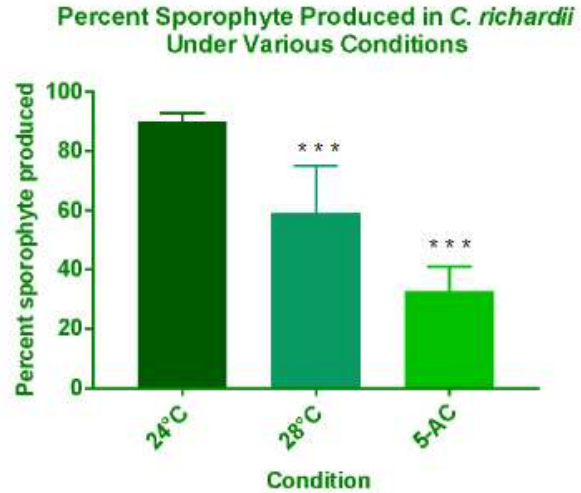

Figure 3: Percentage of hermaphrodites with sporophytes in *Ceratopteris richardii* at room temperature (24°C), elevated temperature (28°C), and with 25μM 5-AC. Error bars represent standard deviation. Asterisks represent statistical significance and p values < .001.

sporophytes produced when gametophytes were treated with 5-AC and or at a temperature of 28℃ compared to the samples at 24℃, which had a much greater percentage of sporophytes.

To determine if Mago nashi may be affected by temperature and 5-AC, an orthologue of Mago nashi was first identified and characterized in *Ceratopteris*. A publically available Expressed Sequence Tag (EST) cDNA library was screened using Basic Local Alignment Search Tool (BLAST).

Species	Orthologue	Accession Number	E-value	Percent Identity
Euphorbia lagascae	mago nashi homolog	O65806.1	4e-95	91%
Oryza sativa	mago nashi homolog	A0A0P0XB7	6e-94	89%
Bos taurus	mago nashi homolog	O23676.1	3e-93	87%
Drosphila melanogaster	mago nashi homolog	P49028.1	3e-86	83%
Danio rerio	mago nashi homolog	Q566Y8.1	6e-86	81%
Homo sapiens	mago nashi homolog	P61326.1	7e-86	81%
Gallus gallus	mago nashi homolog	P505594.2	7e-86	81%
Xenopus laevis	mago nashi homolog	O42149.1	1e-85	81%
Caenorhabditis elegans	mago nashi homolog	P49029.2	2e-84	78%
Dictyostelium discoideum	mago nashi homolog	Q55E21.2	6e-74	69%

Analysis of the putative open reading frame of the sequence showed that the orthologue of Mago nashi identified in *Ceratopteris* is highly conserved and contained all of the essential motifs for functioning such as the binding sites of Tsunagi/Y-14.

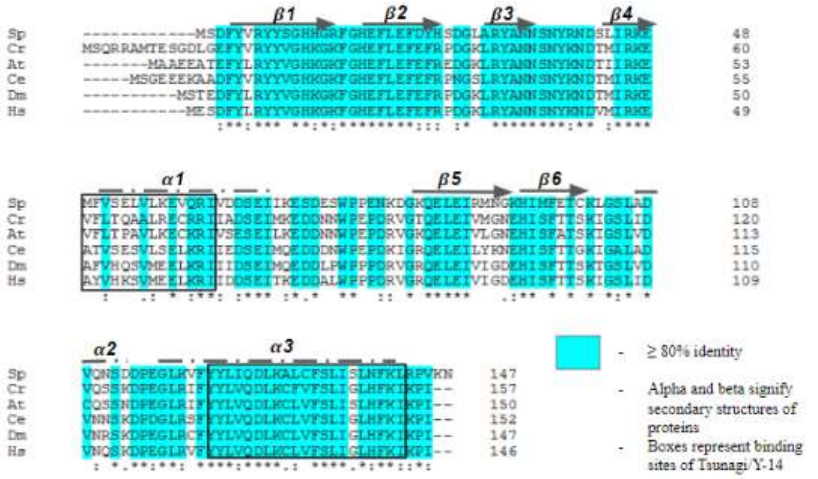

Figure 4: Domain analysis and sequence alignment of various Mago proteins in selected organisms. Blue highlights represent a greater than 80% percent identity. Alpha and beta signify secondary protein structures. Boxes represent binding sites of Tsunagi/Y-14

The amino acid profiles are very conserved between plants, animals and fungi. This assessment is further supported by phylogenetic analysis which showed that Mago nashi gene evolved before the diversification of various kingdoms of organisms and is highly maintained, perhaps because of its critical function in organisms (Figure 5).

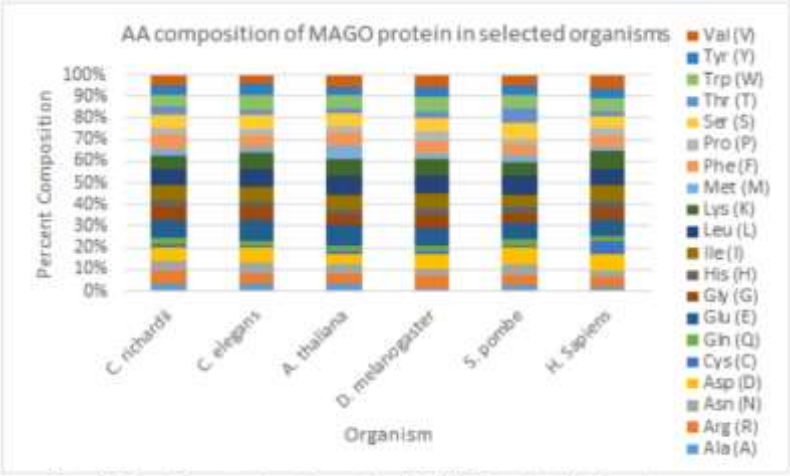

Figure 5: Analysis of percent amino acid composition of the MAGO protein in selected organisms

In *Ceratopteris*, a microarray analysis was performed on germinating spores during the first 48 hours of development. Mago nashi expression can be found in this data set. As seen in Figure 9, the expression of Mago nashi peaked at 12 hrs then declined to lower level at 48 hours.

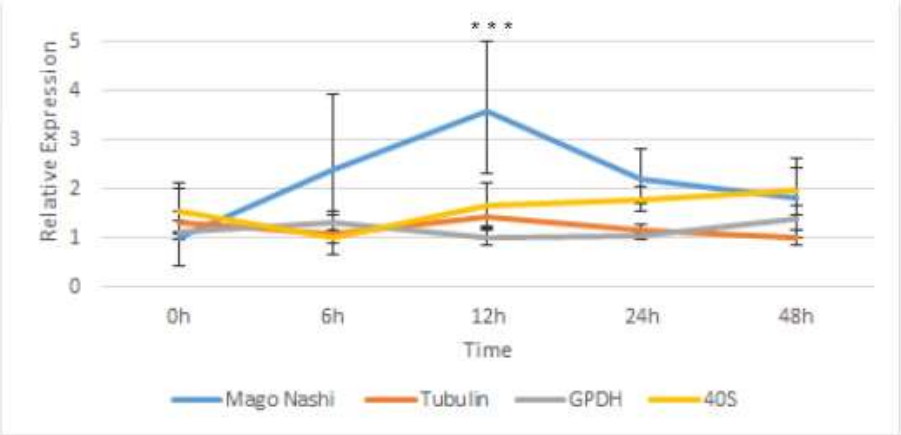

Figure 9: Analysis of microarray data in *Ceratopteris richardii* during first 48hrs of spore development for selected genes. Error bars represent standard deviation, asterisks represent statistical significance.

Using RT-PCR, it was demonstrated that an increase in temperature from 24ºC to 28ºC increases the expression of Mago nashi (Figure 10).

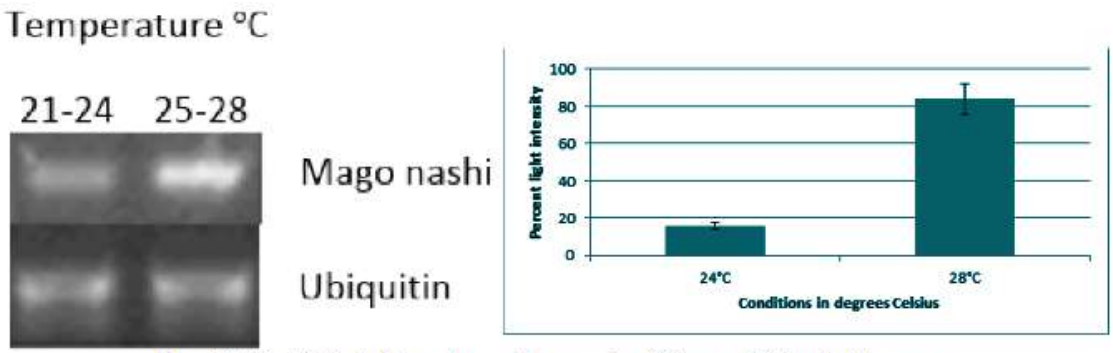

Figure 10: Effect of elevated temperature on the expression of Mago nashi. Gametophytes were grown for 10 days at 21-24 °C or 25-28 °C for 10 days. Total RNA was extracted from tissue, reversed transcribed and amplified by PCR.

Similarly, 5-AC also increased the expression of Mago nashi (Figure 11). The expression of Mago nashi was normalized to a housekeeping gene, β-Tubulin.

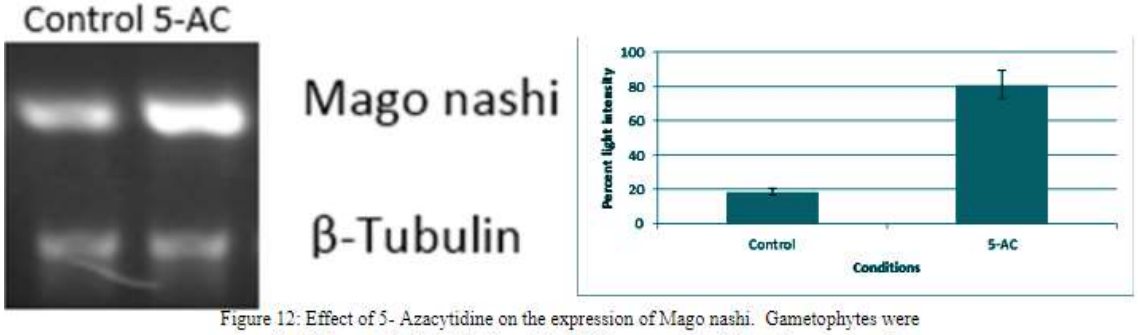

Figure 12: Effect of 5- Azacytidine on the expression of Mago nashi. Gametophytes were grown for 10 days at 21-24 °C 10 days. Total RNA was extracted from tissue, reversed transcribed and amplified by PCR.

In *Ceratopteris*, there are two mutants that have been characterized that resulted in mostly male (*him1*) or hermaphrodites (*her1*). To determine if the mutation that affected sexual differentiation in these mutants affected Mago nashi expression, an RT-PCR was performed. RNA was isolated from these mutants and reverse transcribed into cDNA which was then amplified by PCR with gene specific primers. As seen in Figure 12, there is relatively more expression of Mago nashi in males than hermaphrodite, supporting other data that shows that higher level of Mago nashi correlates with male development.

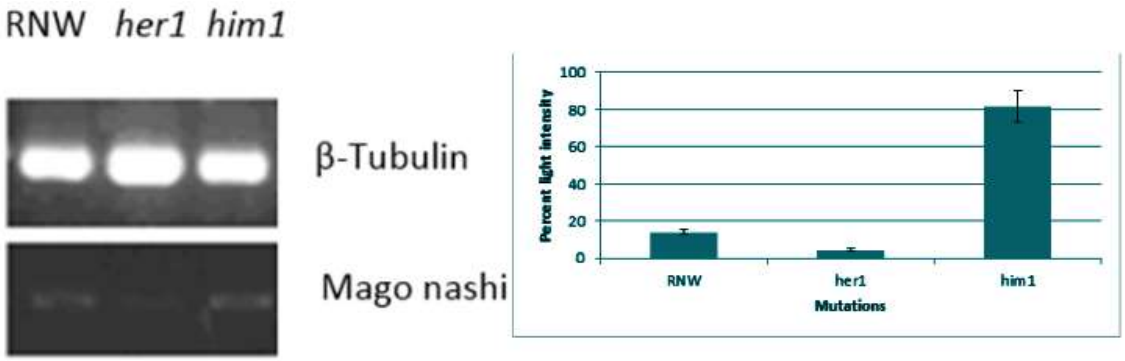

Figure 11: Effect of the *her1* and *him1* mutation on the expression of Mago nashi. Gametophytes were grown for 10 days at 21-24 °C for 10 days. Total RNA was extracted from tissue, reversed transcribed and amplified by PCR.

As seen from this preliminary data, a high level of Mago nashi correlates with male development of gametophytes in *Ceratopteris richardii*. Elevated temperature promotes male differentiation.

Elevated temperature has also been previously shown to be part of the mechanism of demethylation. In a study done in *Arabidopsis thaliana*, under heat stress, DRM2, a type of DNA methyltransferase, and NRPD1 and NRPE1, large subunits of RNA polymerase IV and V, were upregulated (Naydenov *et al*, 2015). This suggests that elevated temperature can lead to methylation of a gene. Despite this observed feature, elevated temperature may have a demethylating effect in other regions which is suggested by the results of this study (Naydenov *et al*, 2015). The results of the study showed that there was an increase in expression of Mago nashi when DNA methylation was inhibited, as well as under heat stress. If elevated temperature results in upregulation of DNA methyltransferase rather than downregulation of it, these findings will not be consistent, which suggests that heat stress has different effects on DNA methylation.

DISCUSSION

Since higher level of Mago nashi has been correlated with male development, a knockdown of Mago nashi may result in more hermaphrodites than males. Future studies assessing the function of the Mago nashi gene in *Ceratopteris richardii* may be completed by

utilizing RNA interference (RNAi). This technique has been very useful in establishing *Ceratopteris richardii* as a model fern since it only requires the incubation of sterilized dry spores with sequence specific double stranded RNA (dsRNA) to reduce the expression of targeted genes (Salmi, *et al*, 2005). If Mago nashi regulates male differentiation, lowering the transcript level of Mago nashi in spores by RNAi should result in more male development

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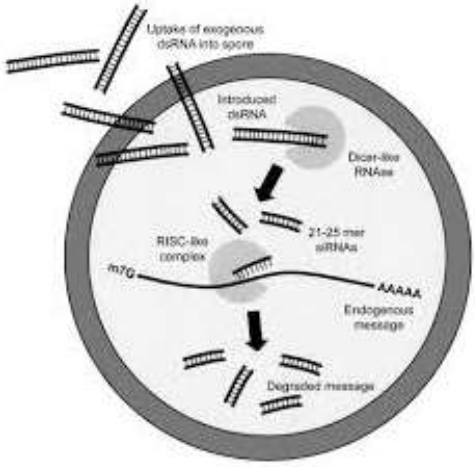

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