Investigating the Use of *Ceratopteris richardii* as a Model Plant for Phytoremediation of Cadmium

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Research Questions:

How does Cd affected the growth and development of *Ceratopteris richardii*?

Can pretreatment of gametophytes with melatonin increase tolerance to Cd?

Can a double mutant resistant to paraquat and glyphosate (*pq45/glt1*) tolerate higher level of cadmium than wild type RNW1?

How does cadmium affect the expression of ASMT gene in wild type RNW1 and the double mutant *pq45/glt1*?

HYPOTHESIS

Since melatonin, N-acetyl-5-metathoxy-tryptamine, has been shown to act directly as an antioxidant as well regulating the activities of antioxidant enzymes, pretreatment of gametophytes with melatonin with increase tolerance to cadmium stress. Melatonin treated gametophytes will show increase development of prothalli, more chlorophyll production and less lipid peroxidation.

Exogenous melatonin has been also shown to improve paraquat tolerance in seed plants. The paraquat tolerant mutant (and glyphosate tolerant) in *Ceratopteris* (*pq45/glt1*) may have higher level of endogenous melatonin and may be tolerant to higher level of cadmium than wild type RNW1.

Objectives

- To characterize the physiological effects of cadmium on prothallial cells of *C. richardii*
- To characterize the effectiveness of melatonin in reducing cadmium phytotoxicity on *C. richardii*
- To compare the level of tolerance to cadmium between wild type RNW1 and double mutant that is resistant to paraquat and glyphosate (*pq45/glt1*)
- To identify an orthologue of ASMT gene in *Ceratopteris richardii*
- To determine if the level of the putative ASMT1 gene is affected by cadmium exposure

INTRODUCTION:

In many parts of the world, heavy metal contamination of soil and water is an emerging problem (Fritioff and Greger, 2006). Although some metals are required in small concentration in biological system, at high concentration, metals can be harmful by producing reactive species, displacing other metal ions, blocking essential functional groups or changing the conformation of biological molecules (Collin and Stotszky 1989).

Cadmium (Cd) is a widely used environmental pollutant (Li *et al* 2016). Pollution of the environment with Cd can occurs from natural and anthropogenic source. In the Earth's crust, Cd concentration is 100-500 µg/L, 100-900 µg/L in lake waters, 900-1100 µg/L in marine sediments (ICdA, 2012). In phosphorous fertilizers, the level can be up to 200 mg/L (Cook and Morrow, 1995).

Cadmium can be found in many household appliances, automobiles and trucks, industrial tools, etc. It is used for luminescent dials in photography, rubber curing and as fungicides (Adriano, 2001). Cd can pollute aquatic systems from agricultural runoff, industrial effluents, fossil fuel combustion, cement and steel production, and municipal solid waste incineration (Li *et al*., 2008; Sun *et al.,* 2008). Since tobacco plant accumulates and concentrate Cd in the leaves, humans can be exposed to this metal through smoking.

Cadmium is one of the most hazardous heavy metals. It is classified as a human carcinogen when taken into the body and can have toxic effect on the kidneys, skeletal and respiratory systems (IPCS 1992, 2005-2007; WHO 2010). Cd can bioaccumulate in plants grown in contaminated soil. It can then enter the food chain via soil-crop systems, leading to potential food safety and human health risks (Liu *et al* 2006; Bernard 2008). In humans, high level of Cd exposure is associated with pulmonary emphysema and Itai-Itai disease (Yeung and Hsu, 2005). It results in bone demineralization because cadmium replaces calcium in the bones.

In plants, cadmium is a highly toxic contaminant that affects many plant metabolic processes. Cd ions can inhibit photosynthesis by inhibiting the synthesis of chlorophyll (Li *et al*., 2008; Sun *et al*, 2008). It can also affect the organization of chloroplasts (Vecchia *et al.,* 2005). Cd can affect the

activities of metabolic enzymes and the composition of cell membrane through the production of Reactive Oxygen Species (ROS, Vecchia, 2005). ROS is not directly produced in the Fenton Reaction but accumulates and eventually caused oxidative stress (Romero-Puertas *et al*, 2002). Under stress conditions (excessive light intensity, heat, salt, cold, drought, and environmental pollutant), large amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced during photosynthesis (Zheng *et al*, 2017). If not detoxified, ROS and RNS can damage chloroplasts and reduces the efficiently of photosynthesis by oxidative stress, leading to cell death. Plants have evolved numerous mechanisms to cope with oxidative stress. They can produce antioxidants (such as ascorbic acid, carotenoids, tocopherol, glutathione, and polyphenols). When Cd accumulates in plant, there is a depletion of the glutathione and inhibition of antioxidant enzymes (Schutzendubel and Polle 2002). There is also a reduction of superoxide dismutase, ascorbate peroxidase, glutathione reductase, and catalase activity (Iannelli *et al*, 2002). This can lead to accumulation of ROS. ROS can results in lipid peroxidation and impairment of the function of the plasma membrane. Cd can alter the permeability of membranes by inhibiting H^+-ATPases, which helps to regulate ionic balances (Janicka-Russak *et al*, 2012). It can increase chromosomal aberrations and affect cell cycle and division (Dal Corso *et al*, 2010). Cd can impede the uptake and translocation of essential elements as well as the water status in plants (Perfus-Barbeoch *et al*, 2002). Different species can accumulate different levels of Cd (Lui *et al*, 2010).

It is very difficult to remediate soil with metal-contaminated soil. Current technologies use soil excavation, landfilling or soil washing with physical or chemical separation of contaminants (Khalid *et al, 2016*). When large area are contaminated, removal of contaminant is difficult. Within an organism, heavy metal cannot be destroyed biologically but can only be transformed from one oxidation state or organic complex to another (Garbisu and Alkorta, 2001). One of the means to remediate contaminated soil is the use of plants (phytoremediation) to extract, sequester and detoxify pollutant (Garbisu *et al*., 2002). Phytoremediation is an ecologically responsible alternative to environmentally destructive physical remediation methods currently practiced (Meagher 2002). Several plants have been proposed for clean-up (phytoremediation) of heavy metal from the soil. Plants have evolved several mechanism that allowed

them to cope with Cd stress. These mechanisms involved immobilization, exclusion, chelation, compartmentation of metal ions and the repair of damaged cell structures (di Toppi and Gabbrielli, 1999; Hall 2002). Cadmium tolerance may be involved in a constitutive detoxification mechanism. To help sequester Cd, plants can release metallthioneins and phytosiderophores **(**Kahlid *et al*, 2017). Cd hyperaccumulator plant *T.caerulescens* have high-level constitutive levels of citric, malic and malonic acids (Boominathan amd Doran 2003). Overexpression of glutathione synthetase has led to increase tolerance of Cd in *B. juncea* (Indian mustard) (Zhu *et al*, 1999). Phytochelatin-based sequestration seems to be essential for constitutive tolerance. There is also an induction of phytochelatin synthesis in *Thlaspi caerulescens* during Cd treatment (Meyer, 2011).

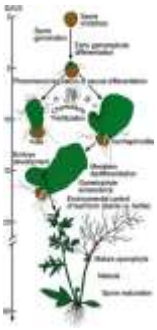
In 1995, melatonin, once believed to be only an animal hormone, but it was identified in plants. In animals, melatonin is a hormone produced by the pineal gland. It plays a role in sleep and the regulation of circadian rhythm. Melatonin has been detected in almost all plant families (Erland, 2015). In plants, melatonin is believed to play a role as an antioxidant and as potent free radical scavenger. Due to its amphiphilic nature, melatonin can enter every sub-cellular compartment (Hardeland, 2005). Studies have shown that melatonin application to leaves protects chlorophyll from degradation during stressful conditions (Arnao & Hernandez-Ruiz, 2008). It is hypothesized that melatonin and serotonin are ancient chemicals evolved in early prokaryotes millions of years ago to cope with the increasing oxygen rich atmosphere (Erland *et al*, 2015). Melatonin is a powerful reducing agent capable of detoxifying a variety of reactive oxygen species.

Melatonin is produced from the amino acid tryptophan involving four essential enzymes: tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin N-acetyltransferase (SNAT), and N-acetylserotonin methytransferase (ASMT) (Li *et al* 2016). ASMT is the final enzyme in sequential pathway that converts N-acetylserotonin to melatonin. It is localized in the cytoplasm and has been shown to upregulated during high light intensity and salt stress (Beyon (2014), Rath (2016). High level of melatonin has been associated with tolerance to salt stress by reducing ROS, lowered lipid peroxidation and enhanced photosynthesis (Gao *et al, 2019).* Several recent studies have demonstrated

that cadmium is an elicitor of the melatonin synthesis pathway in plants (Hyoung-Yool Lee and Kyoungwhan Back 2017). In *Solanum lycopersicum,* exogenous treatment with melatonin can reduce the improve plant biomass and photosynthesis when plants were exposed to cadmium (Debnath *et al*, 2019). Melatonin has been shown to increase in antioxidant activity, increase H^+-ATPase activity and the contents of glutathione and phytochelatins (Hasan *et al*, 2015). Melatonin has been shown to increase play a role in the sub-compartmentalization and transport of cadmium (Hasan *et al*, 2015).

This study explores if the semi-aquatic fern *Ceratopteris richardii* can use to model plant for the phytoremediation of cadmium. *Ceratopteris richardii* is classified under Kingdom Plantae, Division Ptheridophyta, Class Pteridopsida Order Polypodiales and the Family Pteridaceae. It can be found in a variety of tropical areas around the world and is often found as decorative aquarium plant (Banks, 1999). Unlike many ferns, the lifecycle of *Ceratopteris richardii* is relatively rapid. Within 14 days, a mature gametophyte (male or hermaphrodite) can develop from the haploid spore (Hickok, *et al*, 1987). The haploid spores are also excellent for creating and selecting mutations. Since the spores are so small, hundreds of thousands can be mutagenized and screened in a small space. Mutations, regardless of their dominant or recessive character, can be easily identified in the haploid gametophyte. For example, x-ray treated spores resulted in mutations that conferred resistance to the herbicide paraquat (*pq45*) or glyphosate (*glt1*) (Hickok & Tai Chun, 1992). Germinating spores can be develop into males or hermaphrodites and can become sexually mature gametophytes within ten to twelve days to (Chasan, 1992). Both self-fertilization by hermaphroditic gametophytes and cross-fertilization between males and hermaphrodites can occur (Banks, 1999). Within 120 days, *Ceratopteris richardii* can develop from a spore to a mature gametophyte to a young sporophyte that produces new spores (Hickok, *et al*, 1987).

Another important reason for using *Ceratopteris richardii* is its simplicity. The gametophytes of *Ceratopteris richardii* are a two-dimensional layer of cells, making it easy to visualize the effects of experimentation upon cell division and cell elongation (Chasan, 1992).



Life Cycle of *Ceratopteris richardii* (http://cfern.bio.utk.edu)

This study characterizes the responses of *C. richardii* to different amount of cadmium and investigates if melatonin can reduce phytotoxicity. Moreover, this study compares the tolerance between wild type (RNW1) and a double mutant (pq45/glt1) to cadmium. The single mutant *pq45* and *glt1* were isolated by Hickok *et al* but has not been molecularly characterized. The *pq45* single mutant showed no change in the specific activities of superoxide dismutase, catalase, peroxidase, glutathione reductase, dehydroascorbate reductase, and ascorbate peroxidase when grown in presence of paraquat (Carroll *et al.*, 1988). There was also no difference in the levels of ascorbate and glutathione between wild type and mutant. In both wild type and mutant, there was equivalent uptake of paraquat by gametophytes was also equivalent in mutants and wild type. The physiological and molecular basis for tolerance in the *pq45* mutant is unknown. There was no physiological characterization of the *glt1* mutant. This study examines if there is any changes in the melatonin synthesis pathway in the *pq45glt1* double mutant.

METHODOLOGY

A. C-Fern Liquid Media and C-Fern Agar

Liquid C-Fern media (Catalogue# 156782) and C-Fern agar (Catalogue# 156781) were purchased from Carolina Biological and prepared according to manufacturer's instructions. The pH of the media was adjusted to approximately 6.0 with 1M NaOH.

B. Physiological Characterization of gametophytes of *Ceratopteris richardii*

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 (for synchronization). Spores were then plated on C-Fern agar and allowed to develop under continuous white light (100 µE m^{-2} s^{-1}). Light intensity was measured using a digital lux meter (Vici LX-1332B). Germination was measured by the emergence of rhizoids through the spore coat. After 7 days of development, developing gametophytes were exposed to 25 µM $CdCl_2$, 50 µM $CdCl_2$, or 100 µM $CdCl_2$.

Gametophytes were observed for 5-7 days under a compound light microscope with a digital camera. Cell division was assessed by counting the number of cells of the prothalli of the gametophytes.

To determine the amount of chlorophyll produced, 30 gametophytes were counted from each condition in 3 trials, and chlorophyll was extracted for 24 hours at 4˚C in 3ml of 80% acetone. Tissue was pelleted at 14,000 g for 5 minutes and supernatants were measured spectrophotometrically at 663 and 645 nm.

Cell death was assessed using Trypan Blue staining (Sigma Aldrich). Gametophytes were incubated with Trypan Blue for 30 minutes. Gametophytes were then rinsed thoroughly with distilled water. Under a compound microscope, cell death will be analyzed and recorded.

To measure lipid peroxidation, gametophytes were first treated with cadmium as described for 48 hrs. Lipid peroxidation was measured using the Sigma Aldrich Lipid Peroxidation (MDA) Assay Kit (Catalog #MAK085). 10 gametophytes were homogenized in 300 µL of MDA Lysis Buffer containing 3 µL of Butylated hydroxytoluene (BHT). Samples will be centrifuged for 10 minutes at 13,000 g to remove insoluble substances. 200 µL of the supernatant from homogenized samples were placed into a 1.5 ml Eppendorf tube. 600 µL of the TBA solution was added into each tube containing the sample to create the MDA-TBA product. The sample will then incubated at 95 °C for 60 minutes and cooled to room temperature in an ice bath for 10 minutes. Mixtures were placed in cuvettes and analyzed for lipid peroxidation.

To assess the role of melatonin in conferring tolerance to cadmium, 7 days old gametophyte were pretreated with 100 uM of melatonin before the addition of cadmium (as previously described).
ROS levels were measured by treating gametophytes with 5 µM 2'7'-dichlorofluorescin diacetate (DCFH-DA) for 60 minutes. Within the cell, DCFH-DA is converted into DCFH. In the presence of reactive oxygen species, DCFH is then converted to DCF, which is fluorescent. The gametophytes were incubated for 1 hour in the dark at 37°C and then rinsed with distilled water. Gametophytes were observed under a fluorescent microscope (Carl Zeiss-AX10) and images were taken with the Cy5 filter set. They were observed at 490-525 nm. DCFH-DA is converted into DCFH intracellularly. When DCFH is converted into DCF, it fluoresces, indicating the presence of ROS. The integrated density, area of the cell, and the mean fluorescence of the background were measured using Image J. The corrected total cell fluorescence (CTCF) was calculated using the following formula: CTCF = integrated density - (area of selected cell × mean fluorescence of background readings).

C. Molecular Characterization

RNA was isolated using the *Ambion® RNAqueous®-4PCR Total RNA Isolation Kit and MSDS* (Catalog # AM1914). The procedure was followed as specified by manufacturer. RNA was reverse transcribed using the Invitrogen SuperScript® III Platinum® One-Step RT-PCR Kit (Catalog #A11732-020). The procedure followed was that specified by manufacturer. Amplification of the PCR products was analyzed using *Agarose Gel Electrophoresis.* DNA was visualized using 1% agarose gel in 1xTBE solution (89mM Tris Base, 89mM Boric Acid, 2mM EDTA) with SYBR Safe DNA stain and UV light. The primers used to amplify the CrASMT gene were: ACTGCTGTGTCGATGCC (forward) and CAGAATACTGATTCACT (reverse). The Ubiquitin primers used were CrUBQ-F: CCTCACGGGCAAGACCAT TA and CrUBQ-R: ACGGAGAACCAGATGAAGCG. The Actin primers used were CrActin2-F: CGGCATACTGGTGTCATGGT and CrActin2-R: AGCACTGCTTGAATGGCAAC. Conditions for PCR amplification: *Denaturation* at 94 ˚C for 0.5 minutes, *Annealing* at 58 ˚C for 0.5 minutes and *Extension* at 72 ˚C for 2 minutes. The number of cycles ranged from 20-35 cycles.

D. Isolation and Characterization of ASMT gene in *Ceratopteris richardii*.

To identify ASMT gene in *Physcomitrella patens, Selaginella moellendorffii* and *Ceratopteris richardii*, ASMT gene sequences in *Arabidopsis thaliana* were obtained. The Basic Local Alignment Search Tool (BLAST) was used to find similar genes in EST cDNA libraries (www.ncbi.nlm.nih.gov) or in sequenced genomes (www.phytozome.net). Identical genes were filtered out and unique sequences

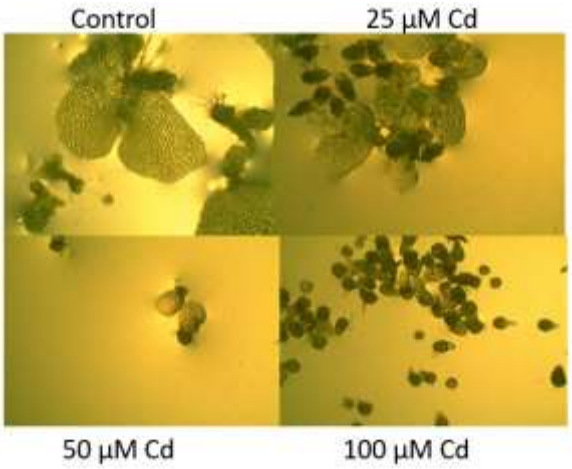
were selected for further analyses. The Open Reading Frame (ORF) Finder was used to translate the cDNA sequences into protein sequences. DNA and translated sequences were analyzed using numerous bioinformatics tools available. Sequence alignment was performed using the ClustalW tool (http://www.clustal.org/). Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis tool (http://www.megasoftware.net/).

Similarities between sequences were determined by aligned protein sequences using a multi-sequence BLASTP and the e-values between sequences were recorded. ClustalW alignments were made for the expressed protein sequences (http://www.clustal.org/). The alignment was color-coordinated based on the physicochemical properties of the amino acid residues.

RESULTS

Characterizing the Effect of Cadmium on Developing Gametophytes

To determine the effect of cadmium on developing gametophytes, spores were allowed to germinate and to develop for 7 days in continuous white light. After 7 days of development, prothallial cells were exposed to different concentrations of cadmium chloride (25, 50 or 100 µM). As seen in Figure 1, exposure to cadmium at even 25 µM significantly inhibited the area development of the prothalli and reduced chlorophyll production.



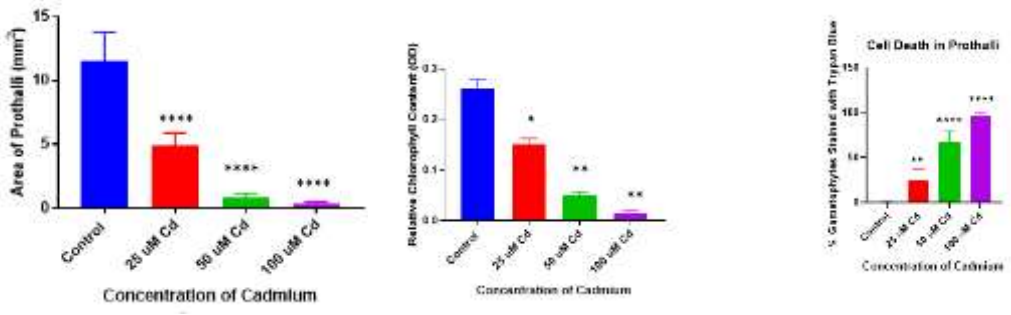
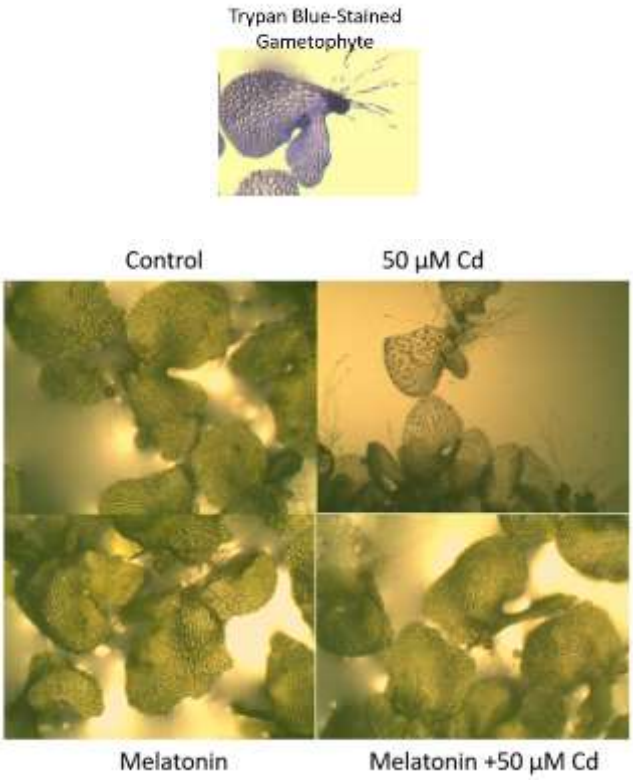


Figure 1. The effect of cadmium of the development of gametophytes of *C.richardii*. A. Area of prothalli and B. Relative amount of chlorophyll. C. Cell Death. Significance was determined with respect to the control using by a one way ANOVA (Dunnet's Multiple Comparison Test). $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.0001$ = ****

Characterizing the Effect of Melatonin on Tolerance to Cadmium

Since a concentration of 50 µM produced evidence of cell death in the prothalli (stained with Trypan Blue), this concentration was used to determine if melatonin can remediate the effect of cadmium. This concentration will also be used to determine if the *pq45/glt1* double mutant was tolerant to cadmium.



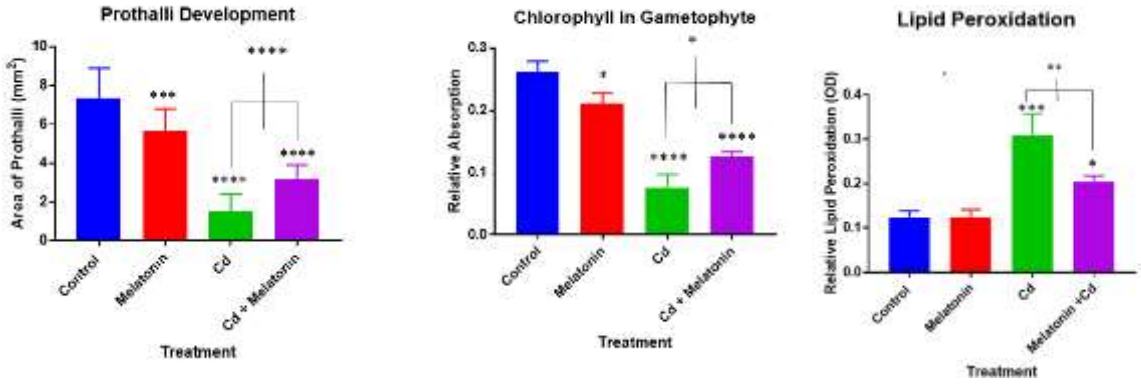
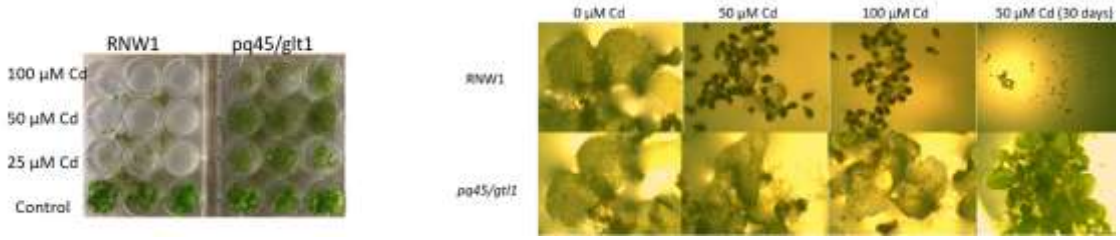


Figure 2: The effect of treatment of gametophytes treated with melatonin and cadmium. Significance was determined using by a one way ANOVA (Tukey's Multiple Comparison Test); P < 0.05 = *, P < 0.01 = **, P<0.001=**, P < 0.0001 = ****

Pretreatment of gametophytes 24 hours with melatonin before expose to cadmium can increase in the development of the prothalli, chlorophyll production and reduce lipid peroxidation. Melatonin can significantly improve tolerance.

Comparing the tolerance of RNW1 (wild type) to the pq45/stl1 double mutant

Previous studies have shown the tolerance of *pq45* and *glt1* to paraquat and glycosate respectively. Several studies in seed plants have shown that an upregulated in the melatonin pathway can improve tolerance to various herbicides. In *Ceratopteris*, there has not been any characterization of the *pq45/glt1* mutant with respect to cadmium or any other pollutants.



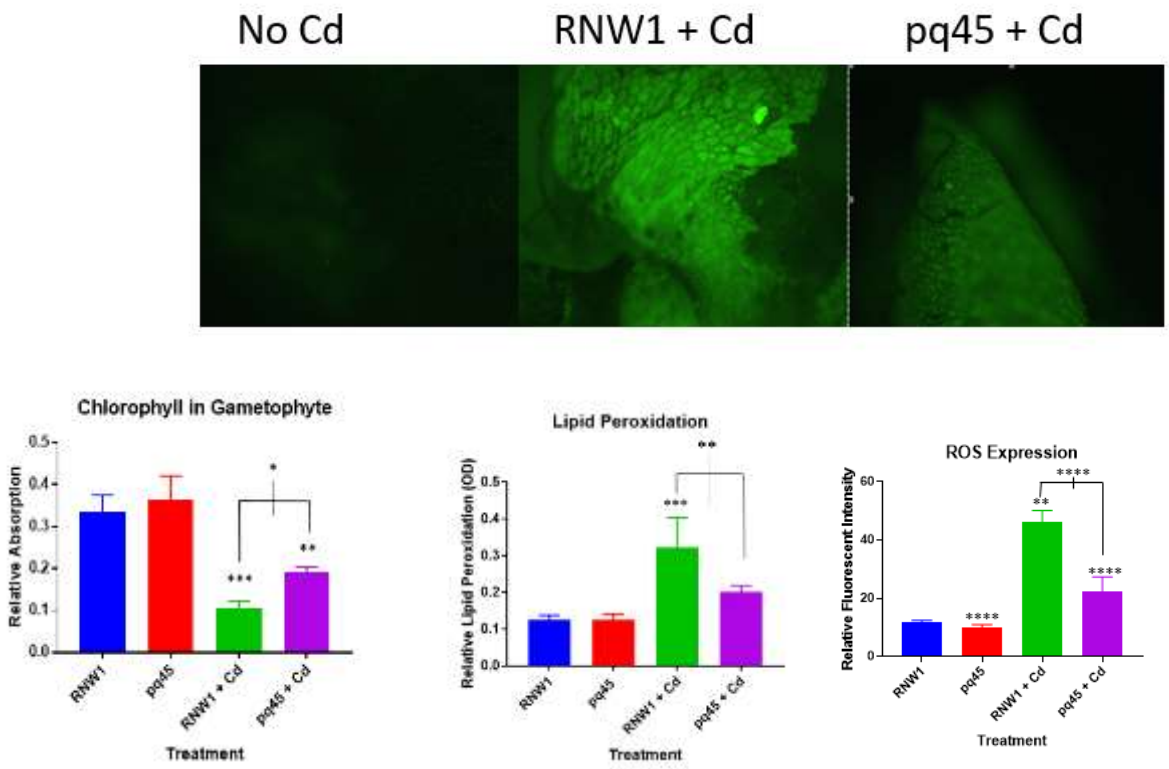


Figure 3: Analyzing the tolerance of *C. richardii* to Cd stress between the wild type and the *pq45/glt1* mutant. Significance was determined using by a one way ANOVA (Tukey's Multiple Comparison Test); P < 0.05 = *, P < 0.01 = **, P<0.001=, P < 0.0001 = ****

Isolation of a partial fragment of ASMT from *Ceratopteris richardii*

To isolate a possible orthologue of ASMT from *Ceratopteris*, degenerate oligonucleotides were designed by aligning ASMT DNA sequences from various plant species. Primers for the Polymerase Chain Reaction (PCR) were designed for regions with the highest conservation.

Figure 4. Designing the PCR primers from conserved regions of ASMT genes.

Using cDNA as template from 10 days old gametophytes, a PCR product was amplified, purified and sequenced.

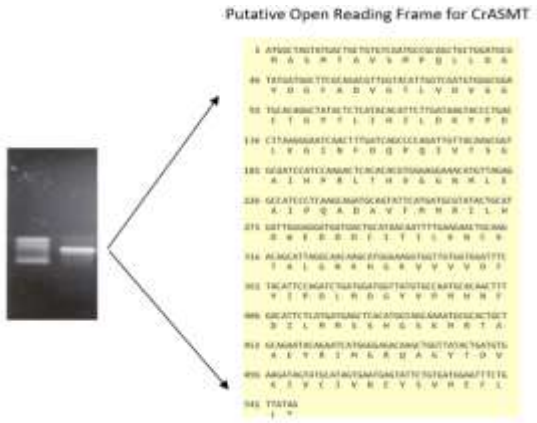
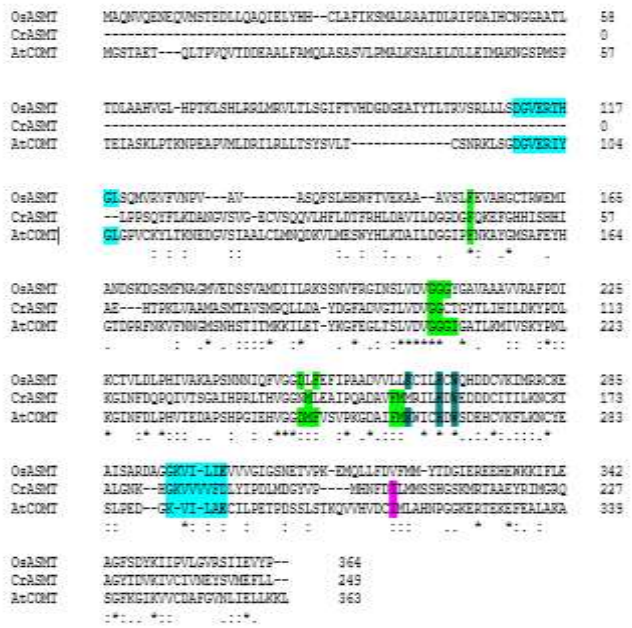


Figure 5. PCR Amplification of putative ASMT orthologue from Ceratopteris and possible Open Reading Frame for the polypeptide.

As seen from the above figure, a continuous open reading frame can be determined from the sequences which shared a 36.96 % identity to anthranilate N-methyltransferase in *A. hypogaea*. Percent identies between *C. richardii* ASMT1 and similar genes in other plants are displayed in the following table.



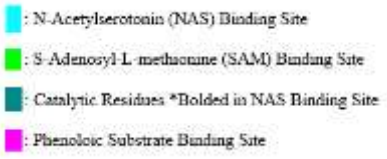


Figure 6: Domain analysis of the ASMT1 gene in *C. richardii*, *A. thaliana*, and *O. sativa*. Putative orthologue sequences were obtained from the TAIR database and from NCBI. Alignment and coloring denotes similarity and key regions of interest within the sequences.

Figure 6 demonstrates that various key regions are conserved between ASMT1 in *C. richardii* and in other previously characterized organisms. The NAS Binding site is a particularly significant sequence as it represents the location in which N-Acetylserotonin, the penultimate molecule in melatonin synthesis, can bind to the ASMT gene allowing for melatonin production to occur (Kang *et al, 2011*). The S-Adenosyl L-methionine Binding Site (SAM) aids in converting N-Acetylserotonin into melatonin by methylation of the hydroxyl group. Catalytic residues shared between the organisms indicate that the catalytic properties of the enzymes are similar in function. Overall, the completed domain analysis indicates that the function of ASMT1 in C. *richardii* is very similar to that of ASMT1 in *Arabidopsis* and *Oryza sativa*. It is possible that the fragment of the gene isolated is more closely related to Caffeic acid O-methyltransferase (COMT). The gene product of COMT has been also shown to methylate N-acetylserotonin into melatonin; that is, it has N-acetylserotonin O-methyltransferase (ASMT) activity. The ASMT activity of COMT was first detected in *Arabidopsis thaliana* COMT (AtCOMT) (Byeon *et al*, 2015). Regardless of whether the fragment of the DNA is from ASMT or COMT, both genes have been shown to be involved in melatonin synthesis.

As expected, ASMT1 evolved before the diversification of seed from seedless plants. Significant sequence divergence among plant species is shown.

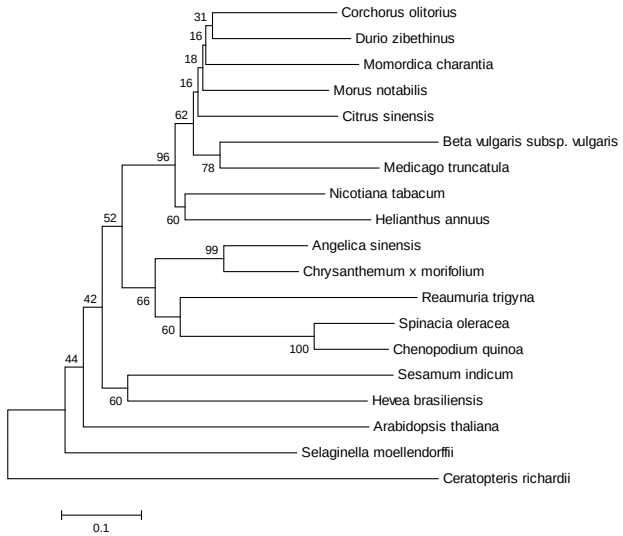


Figure 7: Phylogenetic Tree of ASMT1 in various plant species. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA6.

ASMT from seedless plants occupies a district clade from those of seed plants. It is also possible that divergence in sequences may be due to comparison between ASMT and COMT in species.

Regulation of CrASMT by Cadmium

To determine if the level of transcripts for the putative CrASMT gene was regulated by cadmium, gametophytes were treated with 25 µM of cadmium chloride for 12 hours. RNA was isolated from the gametophytes and reversed transcribed into cDNA, which was then amplified by PCR using gene specific primers. As seen in Figure 8, Cd increases the expression of CrASMT in *Ceratopteris*. Comparison between wild type RNW1 and pq45/glt1 revealed that there is higher level of ASMT in the mutant (without cadmium).

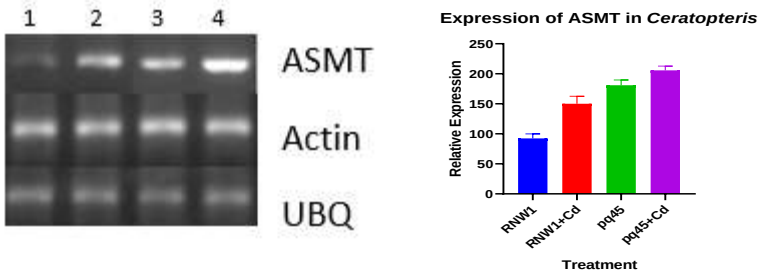


Figure 8. RT-PCR of the expression of ASMT in *Ceratopteris richardii*. Ten day old gametophytes were treated with 25 µM of Cd for 12 hrs. Total RNA was isolated, DNase-treated, reversed transcribed then amplified with primers for ASMT gene. For the controls, housekeeping genes beta actin and ubiquitin (UBQ) was used. Lane 1: RNW1, Lane 2: RNW1 treated with Cd, Lane 3: pq45, Lane 4: pq45 with Cd. The graph displayed above represents relative intensity of the PCR bands denoting gene expression.

This data suggests that there may be constitutive higher level of ASMT in the *pq45/gtl1*. The higher level is further elevated with the addition of cadmium which may be necessary for the increased tolerance.

DISCUSSION

Many recent studies have demonstrated that melatonin in plants plays a crucial role in helping plants respond to increased levels of abiotic stresses such as drought, cold, salt, and extreme temperature stress (Zuo *et al.*, 2014). ASMT is a gene integral to both plants and animals given its function in the melatonin biosynthetic pathway. ASMT is the final gene in the pathway responsible for melatonin production. Melatonin has been found in almost all plant species and is known to have a significant physiological benefit to a given organism due to its ability to serve as an antioxidant and metabolize reactive oxygen species (Tan, 2015, Lahri & Ghosh, 1999). Various stresses that are known to produce ROS have been known to cause less harm on plants when ASMT was upregulated, thus contributing to an increased amount of melatonin as it is produced to serve an antioxidant.

In a study conducted by Park *et al*., 2012, it was demonstrated that transgenic rice plants overexpressing ASMT showed greater tolerance to the herbicide butafenacil. Moreover these plants produced less ROS (hydrogenic peroxide) than non-transformed plants when exposed to butafenacil. As seen in this study, a similar response can be observed in *pq45/glt1* double mutant that showed higher level of CrASMT1 and lower level of ROS when exposed to cadmium. Consistent with previous studies, either endogenous increase of melatonin (from an increase in ASMT) or exogenous addition of melatonin can confer greater tolerance to cadmium stress.

Besides ASMT, upregulation of other genes in the melatonin biosynthesis pathway have been shown to increase tolerance to heavy metals and other abiotic stresses. Increase expression of Seratonin N-acetyltransferase (SNAT) have been shown to increase tolerance to high salinity, heavy metals (including cadmium) and herbicides (Huang et. Al, Park et al, Lee et al.). The melatonin biosynthesis pathway seems extremely important for plants' adaptation to stress. Suppression of ASMT or SNAT has been demonstrated to have detrimental effects on plants on a physiological level (Byeon & Back, 2016). Along with decreased melatonin levels, it was discovered that seedling growth was slowed down.

Exogenous melatonin was then administered to the seedlings, causing growth to partially be restored. ASMT deficient rice also exhibited accelerated senescence in detached flag leaves and significantly reduced yield.

In several plants e.g. rice, multiple isoforms of ASMT gene can found in the genome. These isoforms however showed differential regulation to various chemical treatments. In rice, there are three isoforms of ASMT. Ethephone and salicylic acid has been shown to downregulate the expression of ASMT2 and ASMT3. but did not have an effect on ASMT1 (Park *et al*, 2013). It remains to be determined if there are multiple different isoforms of ASMT gene in *Ceratopteris richardii* and if there isoforms are differentially regulated by cadmium. Moreover other genes in the melatonin synthesis pathway remains to be identified in *C.richardii*.

There are several species of plants within the Genus of *Ceratopteris*. For example, besides *Ceratopteris richardii*, there is *Ceratopteris cornuta*, *Ceratopteris pteridoides*, *Ceratopteris thalictroides*. It has been suggested that *C. thalictroides* actually consists of four cryptic species (*thalictroides, froesii, gaudichaudii, oblongibloba*). In China, *Ceratopteris pteridoides* is an endangered aquatic species. It is found in many ponds, lakes, rivers and ditches in central and southern China (Sun *et al*, 2008). Currently there is only five population of *Ceratopteris pteridoides* (Yu, 1999). One factor that has contributed to the decline is exposure to environmental pollution (Dong *et al*, 2007). In a study by Deng *et al*, 2014, significant reduction of biomass was observed in *C. ptheridoides* at 20 and 40 uM of Cd. There was a significant reduction in the chlorophyll content and decrease in photosynthesis. Cd increased the activity of superoxide dismutase, catalase and peroxidase activities in root and stem. There was also increased lipid peroxidation and membrane damage in the leaves but not in the root (Deng et al, 20014). Understanding the mechanism of tolerance of the *pq45/glt1* mutant of *Ceratopteris richardii* would help develop strains of *C. ptheridoides*, helping the plants survive its polluted environment.

FUTURE STUDIES

Future studies would involve measuring the amount of cadmium in the tissues of RNW1 and *pq45/stl1* mutant to determine if tolerance in the mutant is due to uptake of cadmium or perhaps internal compartmentation of it. The most common analytical procedures for measuring cadmium concentrations in biological samples use the methods of atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic emission spectroscopy (ICP/AES). In addition, the internal level of melatonin can be compared of the mutant to wild type.

Future studies would characterize the nature of the *pq45* and the *stl1* mutations. The effect of cadmium on a single mutation will be compared to double mutant. Moreover, it is important to determine where the mutations for both *pq45* and *stl1* reside on the genome in *Ceratopteris richardii*.

CONCLUSIONS

In this study, it was demonstrated that exogenous application of melatonin can increase tolerance to cadmium stress. Gametophytes treated with melatonin showed increase development of prothalli, more chlorophyll production and less lipid peroxidation in contrast to the untreated gametophytes, supporting the hypothesis. In addition the double mutant pq45/glt1 showed greater tolerance than wild type RNW1 to cadmium. This mutant showed higher level of ASMT expression, the final gene in the melatonin biosynthesis pathway, suggesting that the mutant may have higher level of endogenous melatonin.

REFERENCES

Achuthan, V., DeStefano, J. J., (2015). "Alternative divalent cations (Zn2+, Co2+, and Mn2+) are not mutagenic at conditions optimal for HIV-1 reverse transcriptase activity." BMC Biochemistry, 15, p. 12

Arnao, M. B., Hernandez-Ruiz, J., (2015). "Functions of melatonin in plants: a review." Journal of Pineal Research, 59(2), pp. 133-150

Arnao, M. B., Hernandez-Ruiz, J., (2006). "The Physiological Function of Melatonin in Plants." Plant Signaling and Behavior, 1(3), pp. 89-95

Ayala, A., Munoz, M. F., Arguelles, S., (2014). "Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal." Oxidative Medicine and Cellular Longevity, 2014

Baluska, F., Baroja-Fernandez, E., Pozueta-Romero, J., Hlavacka, A., Etxeberria, E., Samaj, J., (2005). "Endocytic Uptake of Nutrients, Cell Wall Molecules, and Fluidized Cell Wall Portions into Heterotrophic Plant Cells." Plant Endocytosis, pp. 19-35

Banks, J. A., (1999). "Gametophyte Development in Ferns." Annual Review of Plant Physiology and Plant Molecular Biology, 50, pp. 163-186

Barberon, M., Zelazny, E., Robert, S., Conejero, G., Curie, C., Friml, J., Vert, G., (2011). "Monoubiquitin-dependent endocytosis of the Iron-Regulated Transporter 1 (IRT1) transporter controls iron uptake in plants." PNAS, 108(32), pp. E450-E458

Baroja-Fernandez, E., Etxeberria, E., Pozueta-Romero, J., (2012). "Fluid-Phase Endocytosis in Plant Cells." Endocytosis in Plants, pp. 107-122

Byeon, Y., Choi, G., Lee, H., Back, K. (2015). "Melatonin biosynthesis requires N-acetylserotonin methyltransferase activity of caffeic acid O-methyltransferase in rice." Journal of Experimental Botany, 66(21), pp. 6917-6925

Chiang, V. L., Joshi, C. P., (1998). "Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases." Plant Molecular Biology, 37(4), pp. 663-674

Chen, X., Irani, N. G., Friml, J. (2011). "Clathrin-mediated endocytosis: the gateway into plant cells." Current Opinion in Plant Biology, 14(6), pp. 674-682

Clemens, S., (2002). "Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants." Biochimie, 88(11), pp. 1707-1719

Colangelo, E. P., Lou-Guerinot, M., (2006). "Put the metal to the petal: metal uptake and transport throughout plants." Current Opinion in Plant Biology, 9(3), pp. 322-330

Conner, S. D., Schmid, S. L., (2003). "Regulated portals of entry into the cell." Nature, 422, pp. 37-44

Cvjetko, P., Zovko, M., Balen, B., (2014). "Proteomics of heavy metal toxicity in plants." Archives of Industrial Hygiene and Toxicology, 65(1), pp. 1-18

Das, P., Samantaray, S., Rout, G. R., (1997), "Studies on cadmium toxicity in plants: a review." Environmental Pollution, 98(1), pp. 29-36
Deng, G., Li, M., Li, H., Yin, L., Li, W. (2014). "Exposure to cadmium causes declines in growth and photosynthesis in the endangered aquatic fern Ceratopteris pteridoides." Aquatic Botany, pp. 23-32

Dhir, B., Sharmila, P., Saradhi, P. P., Sharma, S., Kumar, R., Mehta, D., (2011). "Heavy metal induced physiological alterations in Salvinia natans." Ecotoxicology and Environmental Safety, 74(6), pp. 1678-1684

Erland, L. A., Chattopadhyay A., Jones, A. M., Saxena, P. K., (2016). "Melatonin in Plants and Plant Culture Systems: Variability, Stability and Efficient Quantification." Frontiers in Plant Science, 7, p. 1721

Erland, L. A., Murch, S. J., Reiter, R. J., Saxena, P. K., (2015). "A new balancing act: The many roles of melatonin and serotonin in plant growth and development." Plant Signaling & Behavior, 10(11), e1096469

Godt, J., Scheidig, F., Grosse-Siestrup, C., Esche, V., Brandenburg, P., Reich, A., Groneberg, D. A., (2006). "The toxicity of cadmium and resulting hazards for human health." Journal of Occupational Medicine and Toxicology, 1(1), pp. 22

Gupta, M., Devi, S., Singh, J., (1992). "Effects of Long-Term Low-Dose Exposure to Cadmium during the Entire Life Cycle of Ceratopteris thalictroides, a Water Fern." Archives of Environmental Contamination and Toxicology, 23(2), pp.184-189

Hall, J. L., Williams, L. E., (2003). "Transition metal transporters in plants." Journal of Experimental Botany, 54(393), pp. 2601-2613

Hardeland, R., (2016). "Melatonin in Plants - Diversity of Levels and Multiplicity in Functions." Frontiers in Plant Science, 7, p. 198

Hardeland R., (2016). "Melatonin: Another Phytohormone?" Research & Reviews: Journal of Botanical Sciences, 5(2)

Hasan, M. K., Ahammed, G. J., Yin, L., Shi, K., Xia, X., Zhou, Y., Yu, J., Zhou, J., (2015). "Melatonin mitigates cadmium phytotoxicity through modulation of phytochelatins biosynthesis, vacuolar sequestration, and antioxidant potential in Solanum lycopersicum L." Frontiers in Plant Science, 6, p. 601

Hickok, L. G., Warne, T. R., Fribourg, R. S., (1995). "The biology of the Fern Ceratopteris and its use as a model system." International Journal of Plant Sciences, 156(3), pp.332-345

Hou, G. C., Hill, J. P., (2004). "Developmental anatomy of the fifth shoot-borne root in sporophytes of Ceratopteris." Planta, 219(2), pp. 212-220

Johnson, G., Renzaglia, K. (2008). "Embryology of Ceratopteris richardii (Pteridaceae, tribe Ceratopterideae), with emphasis on placental development." Journal of Plant Research, 121(6), pp. 581–592

Kaczmarczyk, A., Hochstrasser, R., Vorholt, J. A., Francez-Charlot, A., (2015). "Two-tiered

histidine kinase pathway involved in heat shock and salt sensing in the general stress response of Sphingomonas melonis Fr1." Journal of Bacteriology, 197(8), pp. 1466-1477

Kim, Y., Lee, Y. N., Oh, Y. J., Hwang, I., Park, W. J., (2007). "What is the role of melatonin in plants? Review on the current status of phytomelatonin research." JNBT, 4(1), pp. 9-14

Kolar, J., Machackova, I., (2005). "Melatonin in higher plants: occurrence and possible functions." Journal of Pineal Research, 39(4), pp. 333-341

Kolodziejczyk, I., Posmyk, M. M., (2016) "Melatonin - a new plant biostimulator?" Journal of Elementology, 21(4), pp. 1187, 1198

Li, M., Hasan, K., Li, C., Ahammed, G., Xia, X., Shi, K., Zhou, Y., Reiter, R., Yu, J., Xu, M., Zhou, J. (2016). "Melatonin mediates selenium-induced tolerance to cadmium stress in tomato plants." Journal of Pineal Research, 61(3), pp. 291-302

Li, W., Lu, J., Lu, K., Yuan, J., Huang, J., Du, H., Li, J., (2016). "Cloning and Phylogenetic Analysis of Brassica napus L. Caffeic Acid O-Methyltransferase 1 Gene Family and Its Expression Pattern under Drought Stress." PLOS One, 11(11), e0165975

Limson, J., Nyokong, T., Daya, S., (1998). "The interaction of melatonin and its precursors with aluminum, cadmium, iron, lead, and zinc: An adsorptive voltammetric study." Journal of Pineal Research, 24(1), pp. 15-21

Liu, Y., Liu, K., Li, Y., Yang, W., Wu, F., Zhu, P., Zhang, J., Chen, L., Gao, S., Zhang, L., (2016). "Cadmium contamination of soil and crops is affected by intercropping and rotation systems in the lower reaches of the Minjiang River in south-western China." Environmental Geochemistry and Health, 38(3), pp. 811-820

Malgieri, G., Palmieri, M., Esposito, S., Maione, V., Russo, L., Baglivo, I., de Paola, I., Milardi, D., Diana, D., Zaccaro, L., Pedone, P. V., Fattorusso, R., Isernia, C., (2014). "Zinc to cadmium replacement in the prokaryotic zinc-finger domain." Metallomics, 6(1), pp. 96-104

Martelli, A., Rousselet, E., Dycke, C., Bouron, A., Moulis, J. M., (2006). "Cadmium toxicity in animal cells by interference with essential metals." Biochemie, 88(11), pp. 1807-1814

Murch, S. J., Campbell, S. S. B., Saxena, P. K., (2001). "The role of serotonin and melatonin in plant morphogenesis: Regulation of auxin-induced root organogenesis in in vitro-cultured explants of St. John's Wort (Hypericum perforatum L.)." In Vitro Cellular & Development Biology - Plant, 37(6), pp. 786-793

Pévet, P. (2003). "Melatonin in animal models." Dialogues in Critical Neuroscience, 5(4), pp. 343-352

Plackett, A. R. G., Huang, L., Sanders, H. L., Langdale, J. A., (2014). "High-Efficiency Stable Transformation of the Model Fern Species Ceratopteris richardii via Microparticle Bombardment." Plant Physiology, 165(1), pp.3-14
"Preface: Melatonin and plants.", Journal of Experimental Botany, 2015, 66(3), pp. 625-626

Rathinasabapathi, B., (2006). "Ferns represent an untapped biodiversity for improving crops

for environmental stress tolerance.", 172(3), pp. 385-390

Reiter, R. J., Tan, D., Zhou, Z., Cruz, M. H., Fuentes-Broto, L., Galano, A., (2015). "Phytomelatonin: assisting plants to survive and thrive." Molecules, 20(4), pp. 7396-7437

Renzaglia, K., Warne, T. (1995). "Ceratopteris: an ideal model system for teaching plant biology." International Journal of Plant Science, 156(3), pp. 385-392.

Renzaglia, K. S., Warne, T. R., Hickok, L. G., (1995). "Plant Development & the Fern Life Cycle Using Ceratopteris richardii." The American Biology Teacher, 57(7), pp. 438-442

Salmi, M. L., Morris, K. E., Roux, S. J., Porterfield, D. M., (2007). "Nitric Oxide and cGMP Signaling in Calcium-Dependent Development of Cell Polarity in Ceratopteris richardii." Plant Physiology, 144(1), pp. 94-104

Salmi, M. L., Bushart, T. J., Stout, S. C., Roux, S. J., (2005). "Profile and Analysis of Gene Expression Changes during Early Development in Germinating Spores of Ceratopteris richardii" Plant Physiology, 138(3), pp. 1734-1745

Sessa, E. B., Banks, J. A., Barker, M. S., Der, J. P., Duffy, A. M., Graham, S. W., Hasebe, M.,

Langdale, J., Li, F., Marchant, D. B., Pryer, K. M., Rothfels, C. J., Roux, S. J., Salmi, M. L., Sigel, E. M., Soltis,

D. E., Soltis, P. S., Stevenson, D. W., Wolf, P. G., (2014) "Between Two Fern Genomes." GigaScience, 3(15).

Sonmez, B., Yanik-Yildirim, K. C., Wood, T. K., Vardar-Schara, G., (2014). "The role of substrate binding pocket residues phenylalanine 176 and phenylalanine 196 on Pseudomonas sp. OX1 toluene o-xylene monooxygenase activity and regiospecificity." Biotechnology and Bioengineering, 111(8), pp. 1506-1512

Strain, E., Hass, B., Banks, J. A., (2001). "Characterization of Mutations That Feminize Gametophytes of the Fern Ceratopteris." Genetics, 159(3), pp. 1271-1281

Szafranska, K., Szewczyk, R., Maria-Janas, K., (2014). "Involvement of melatonin applied to Vigna radiata L. seeds in plant response to chilling stress." Central European Journal of Biology, 9(11), pp. 1117-1126

Szafranska, K., Reiter, R. J., Posmyk, M., (2017). "Melatonin Improves the Photosynthetic Apparatus in Pea Leaves Stressed by Paraquat via Chlorophyll Breakdown Regulation and its Accelerated de novo Synthesis.", 8, p. 878

Tan, D., Hardeland, R., Manchester, L. C., Korkmaz, A., Ma, S., Rosales-Corral, S., Reiter, R. J., (2012). "Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science." Journal of Experimental Botany, 63(2), pp. 577-597

Terwilliger, T. C., Wang, J. Y., Koshland, D. E., (1986). "Surface structure recognized for covalent modification of the aspartate receptor in chemotaxis." PNAS, 83(18), pp. 6707-6710

Warne, T. R., Vogelien, D. L., Hickok, L. G., (1995). "The Analysis of Genetically and

Physiologically Complex Traits Using Ceratopteris: A Case Study of NaCl-Tolerant Mutants." International Journal of Plant Sciences, 156(3), pp. 374-384

Weeda, S., Zhao, X., Ndip, G., Guo, Y., Buck, G. A., Fu, C., Ren, S., (2014). "Arabidopsis Transcriptome Analysis Reveals Key Roles of Melatonin in Plant Defense Systems." PLOS One, 9, e93462

Yang, M., Zhang, F., Wang, F., Dong, Z., Cao, Q., Chen, M., (2015). "Characterization of a Type 1 Metallothionein Gene from the Stresses-Tolerant Plant Ziziphus jujuba." International Journal of Molecular Sciences, 16(8), pp. 16750-16762
Zheng, X., Tan, D. X., Allan, A. C., Zuo, B., Zhao, Y., Reiter, R. J., Wang, L., Wang, Z., Guo, Y.,

Zhou, J., Shang, D., Li, Q., Han, Z., Kong, J., (2017). "Chloroplast biosynthesis of melatonin and its involvement in protection of plants from salt stress." Scientific Reports, 7, 41236

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