Spring 2020

The Coordinate Regulation of Digestive Enzymes in the Pitchers of Nepenthes ventricosa

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2020

Abstract

Many species of plants have adopted carnivory as a way to obtain supplementary nutrients from otherwise nutrient deficient environments. One such species, *Nepenthes ventricosa,* is characterized by a pitcher shaped passive trap. This trap is filled with a digestive fluid that consists of many different digestive enzymes, the majority of which seem to have been recruited from pathogen resistance systems. The present study attempted to determine whether the introduction of a prey stimulus will coordinately upregulate the enzymatic expression of a chitinase and a protease while also identifying specific chitinases that are expressed by *Nepenthes ventricosa***.** We were able to successfully clone NrCHIT1 from a mature *Nepenthes ventricosa* pitcher via a TOPO-vector system. In order to asses enzymatic expression, we utilized RT-qPCR on pitchers treated with chitin, BSA, or water. Unfortunately, we were unable to draw definitive conclusions about the coordinate expression of the digestive enzymes.

Introduction

Evolution of Carnivory

Carnivory is an extraordinary adaptation through which certain plants can digest insects and small vertebrates in order to obtain supplemental nutrients. This ability enables the survival of carnivorous plants in the nutrient deficient environments that they typically inhabit. However, carnivory comes at a cost. The traps used to capture prey are actually epiascidate leaves. These traps have a significantly lower rate of photosynthesis than the standard leaf. This carbon cost is large but worth the risk; nutrients absorbed through carnivory often surpass those that are sacrificed by having these modified leaves (Pavlovič and Saganová, 2015). Additionally, this carbon cost is offset by the high sunlight, high moisture, environments that many carnivorous plants inhabit (Ellison and Gotelli, 2009).

Genetic analysis of specific species has led researchers to believe that carnivory has evolved via a repurposed pathogen-resistance system (Schulze et al., 2012; Hatano et al., 2008). The molecules and mechanisms utilized in the pathogen-resistance systems of non-carnivorous plants are extremely similar to that seen in carnivory. Specifically, proteins classified as pathogenesis-related (PR) proteins are often employed during plant carnivory. PR proteins such as chitinases, phosphatases, nucleases, peroxidases, and phospholipases that share sequence identity with pathogenesis-related proteins found in non-carnivorous plants were identified in the carnivorous plant *Dionaea muscipula* (Schulze et al., 2012). Genetic analysis of *Nepenthes* pitcher fluid further supports the hypothesis that carnivory evolved from a pathogen-resistance system because of the intense similarities between the patterns of genes expressed in the pitcher fluid and the pathogen-resistance system of non-carnivorous plants (Hatano and

Hamada, 2012). PR proteins found in the pitcher fluid of *Nepenthes* included glucanases, xylosidases, and peroxidases. Similar to responses against pathogens in normal plants, peroxidase expression increases in response to a prey stimulus, mirroring an induced defense system (Hatano and Hamada, 2012).

The Nepenthes Genus

As an example of convergent evolution, carnivory has developed in various distinct lineages. This has resulted in a diversity of traps that utilize several different mechanisms for prey capture including suction, adhesion, snapping, and pitfall (Ellison and Gotelli, 2009). The *Nepenthes* genus is characterized by a pitcher shaped passive trap; these modified leaves consist of a slippery peristome that attracts the insects, a waxy inside wall that prevents escape, and a fluid-filled cup that facilitates digestion (Figure 1). The peristome is encircled by extra-floral nectaries that encourage insects to approach the trap (Owen and Lennon, 1999). Once they have been lured by the promise of nectar, the insects are able to climb down into the pitcher. The inside wall of the pitcher is lined with a dense layer of epicuticular waxy scales (Figure 2). These scales overlap each other, starting at the peristome and working their way towards the center of the pitcher. This pattern is the result of anisotropic growth and, though it allows the insects to climb into the pitcher, they have great difficulty climbing in the opposite direction. This, combined with the high hydrophobicity of the waxy inside wall, makes it almost impossible for the insects to escape (Moran and Clarke, 2010).

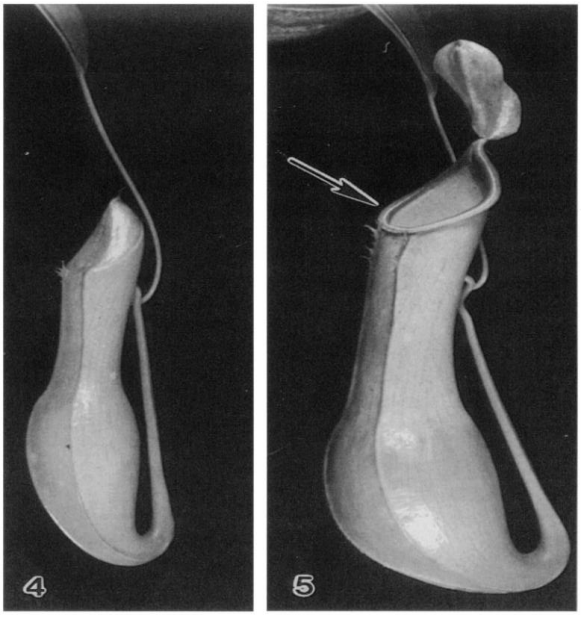

Figure 1. Unopened and opened *Nepenthes* pitchers. The inside of the pitcher remains sterile until it has matured enough to open. Arrow points to peristome. (Adapted from Owen and Lennon, 1999)

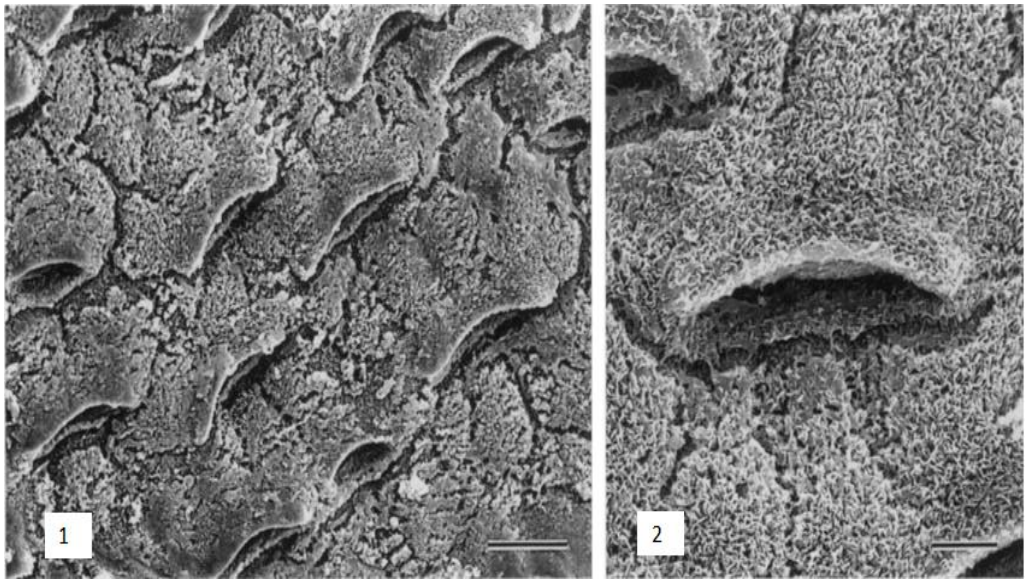

Figure 2. The inner epidermis of a *Nepenthes alata* pitcher. The curved scales lining the upper inside portion of the pitcher face downward – making it more difficult for insects to escape. Images were acquired via scanning electron microscopy. Bar = 10 μm. (Adapted from Owen and Lennon, 1999)

There are some distinct variations between the pitcher traps of *Nepenthes* that are the result of particular adaptations. For instance, *Nepenthes ampullaria* do not rely on insects as their primary source of nutrients. Rather, they gain a large quantity of their required nitrogen from the leaf litter that is prevalent in their environment. The pitchers of *Nepenthes ampullaria* exist close to the ground in tightly packed clusters, which exposes them to a high degree of leaf litter. As a result, the pitcher fluid is significantly less viscous than other *Nepenthes* species (Moran et al., 2003). The viscosity of a pitcher's fluid is highly affected by its polysaccharide content. *Nepenthes* that are primarily insectivores secrete polysaccharides into the fluid to assist with prey capture (Bazile et al., 2015). Those that are less insectivorous will often lack characteristics attractive to insects (Gaume et al., 2016). Depending on the type of insect the pitcher is attempting to attract, *Nepenthes* pitchers can exhibit a few other adaptations. Those that target flying insects often have wide, conical pitchers, while those attracting termites will have more narrow pitchers. Plants attempting to attract ants will often secrete an extrafloral nectar in order to bait the insects (Gaume et al., 2016). The majority of *Nepenthes* pitchers will develop in a closed conformation, opening only after they have filled with fluid and reached maturity (Figure 1) (Owen and Lennon, 1999).

Once the pitchers open, the pitcher fluid becomes available to a wide variety of bacteria that begin to populate the trap. Bacteria from 100 families and 195 genera inhabit the microbial community of *Nepenthes* pitcher fluid but those found in one pitcher may vary significantly from that of another (Takeuchi et al., 2015). The most common bacteria found in *Nepenthes* pitchers are those in the phyla *Bacteroidetes, Actionbacteria,* and *Proteobacteria.* These

bacteria are thought to aid in the digestive process, as some possess proteolytic and chitinolytic activity (Chan et al., 2016). Particular species of these bacteria have even been found to secrete chitinases and lipases into *Nepenthes* pitcher fluid. These enzymes were found to be active under the acidic conditions of the pitchers, which supports the notion that the bacteria are promoting digestion and therefore exist in a symbiotic relationship with the carnivorous plant (Morohoshi et al., 2011).

Nepenthes Pitcher Fluid

The fluid within a *Nepenthes* pitcher contains many enzymes that are secreted into the fluid via digestive glands located at the bottom of the pitcher. Once prey is detected, these glands not only deliver digestive enzymes into the pitcher, but also absorb the newly available nutrients. The exact composition of the pitcher fluid varies dramatically, however over 30 enzymes have been identified in *Nepenthes* to serve a digestive function (Rottloff et al., 2016)

A diversity of proteases are prevalent in the pitcher fluid of *Nepenthes*, suggesting that their involvement in digestion is essential. A novel aspartic protease dubbed “nepenthesin” was first identified in *Nepenthes alata* (An et al., 2002). Nepenthesins are pepsin-type aspartic proteases that are distinguished by a conserved plant-specific insert (PSI) and a low sequence homology to other proteases. The PSI is a conserved amino acid sequence found in plant proteinases that is about 100 amino acids long (An et al., 2002). Nepenthesins isolated from *Nepenthes gracilis* were found to perform optimally in the acidic pitcher fluid, but also remain stable throughout fluctuating temperature and pH conditions. This stability is attributed to the protein’s ability to form multiple disulfide bridges as a result of the prevalent cysteine residues

(Athauda et al., 2004). Stephenson and Hogan (2006) successfully cloned a cysteine protease (NvCP1) and an aspartic protease (NvAP1) from *Nepenthes ventricosa*. The aspartic protease was found to have high homology to aspartic proteinases such as Nepenthesin and contains the conserved PSI that distinguishes theses plant aspartic proteases.

Pitcher fluids also contain many digestive enzymes other than proteases, such as lipases, nucleases, and chitinases (Rottloff et al., 2016). Though there has been lipase activity reported, it is uncertain whether this is the result of plant produced enzymes or the microbes that inhabit the fluid (Morohoshi et al., 2011). Part of a sequence of a known lipase has been isolated from the pitcher tissue of *Nepenthes mirabilis*, suggesting that lipases are secreted into the pitcher fluid (Rottloff et al., 2016). A ribonuclease (NvRN1) has been cloned from *Nepenthes ventricosa*, containing the five conserved domains usually associated with S-like ribonucleases (Stephenson and Hogan, 2006). There have also been many chitinases isolated from *Nepenthes* species. A description of these follows.

Classification of Chitinases

Chitinases are a diverse collection of enzymes that each have a catalytic glycoside hydrolase domain, which severs glycosidic bonds. This glycoside hydrolase domain divides chitinases into two families: glycosyl hydrolase family 18 (GH18) and glycosyl hydrolase family 19 (GH19). Additionally, Chitinases are usually separated into five classes. However, some sources separate them into seven (Grover, 2012). Of these classes, III and V exhibit a GH18 domain while I, II, IV, VI, and VII contain a GH19 domain. Each class is further distinguished by the presence, or absence, of a chitin-binding domain. The classes that possess a chitin-binding

domain (I, IV, and V) exhibit a highly conserved, cysteine rich N-terminal region that is about 40 amino acids long (Grover, 2012).

Class I chitinases have two subclasses: 1a and 1b. These enzymes are highly similar but serve significantly distinct functions. Class I chitinases that exhibit a C-terminal extension are classified as 1a. This C-terminal extension starts with Gly-Leu-Leu and serves to localize the enzyme in the vacuole (Esaka et al., 1990). These class 1a chitinases are thought to be housekeeping enzymes with the primary purpose of defense and maintenance of the pitcher tissue. On the other hand, class 1b chitinases are thought to be primarily for prey digestion. Class 1b lacks the C-terminal extension and, as a result, are secreted into the pitcher fluid where they have access to the prey components. As a whole, class I chitinases are thought to have originated from pathogen resistance proteins that eventually resulted in a subclass adapted for carnivory (Renner and Specht, 2012).

Chitinases as a Digestive Component

Chitinases are of particular note in the study of carnivorous plants because they appear to be utilized for both digestive and defensive purposes. Insect exoskeletons are made of chitin, but so are the cell walls of most fungi. Chitin digestion therefore serves many purposes. It degrades potentially harmful fungal cells, breaks down tough prey components, and provides a valuable portion of nitrogen from the chitin itself (Eilenberg et al., 2006). The fact that chitin can signal both a hostile intruder and prey became apparent in a study that found that a chitin stimulus resulted in a *Nepenthes* pitcher secreting antifungal naphthoquinones (Eilenberg et al.,

2010). These observations further support the notion that carnivory is an evolved form of pathogen resistance.

Several studies have sought to investigate the chitinase content on *Nepenthes* pitchers. Eilenberg et al. (2006) identified the basic class I chitinases NkCHIT1b and NkCHIT2b from *Nepenthes khasiana*. However, it is unlikely that a basic chitinase would be primarily involved in the highly acidic process of digestion. NkCHIT1b exhibited a proline rich hinge, which indicates that it localizes in the extracellular space. On the other hand, NkCHIT2b contains a C-terminal extension that directs the protein to the vacuole, therefore eliminating it as a digestive enzyme. As such, NkCHIT2b in constitutively expressed despite a chitin stimulus, while NkCHIT1b expression increases in response to chitin (Eilenberg et al., 2006). Rottloff et al. (2011) found the class III acid endochitinase NrChit1 in *Nepenthes rafflesiana*, and Hatano and Hamada (2012) discovered the chitinases NaCHIT3 (class III) and NaCHIT1 (class IV) in *Nepenthes alata*. These are proposed to play a role in both digestion and the prevention of fungal growth. The class III chitinase, NaCHIT3, has been reported to perform optimally under acidic conditions (pH 3.9), and prefers polymeric substrates rather than oligomeric substrates. This suggests that NaCHIT3 is well suited as a digestive enzyme because it can function in the acidic pitcher fluid and it can break down the long polymers of chitin found in the exoskeletons of insects. (Ishisaki et al., 2012a). It has been proposed that NaCHIT1 breaks down the oligomers produced by NaCHIT3, due to NaCHIT1's affinity for smaller chitin fragments and a higher pH similar to that of a pitcher post-capture (Ishisaki et al., 2012b). Recently, Filyushin et al. (2019) identified fifteen chitinases (classes I-V) from the pitchers and petioles of *Nepenthes* sp. This is the first

study to isolate class II and V chitinases from the *Nepenthes* genus. However, little research has occurred to identify the chitinases of *Nepenthes ventricosa.*

Induction of Digestion

The induction and regulation of the digestive enzymes in *Nepenthes* pitcher fluid is an ongoing topic of investigation. Active trapping carnivorous plants, such as the Venus flytrap, induce digestion in response to electrical signals that are physically stimulated by the prey entering the trap (Bemm et al., 2016). These electrical signals have not been found in plants that have pitcher traps. This would suggest that these plants instead rely on chemical signaling to indicate the presence of prey. However, there is evidence that suggests that some proteases are secreted at a standard level independent of a prey stimulus. Stephenson and Hogan (2006) found that an aspartic protease was active in the pitcher fluid when it first opened and that activity did not decrease over time. In contrast, some studies have demonstrated that aspartic proteases are upregulated in response to a prey stimulus (An et al., 2002).

Certain studies have focused on investigating chitinase induction through varying prey stimuli. In *Nepenthes raffelsianna,* the chitinase NrChit1 was expressed in response to *Drosophila* that were introduce to the pitcher (Rottloff et al., 2011). As previously discussed, certain chitinases such as NkCHIT2b do not undergo increased expression when prey is detected. These chitinases are thought to be primarily for pathogen resistance systems (Eilenberg et al., 2006). Eilenberg et al. (2006) illustrated the upregulation of certain chitinases in response to an injection of colloidal chitin. Chitin treatments have also been shown to induce antimicrobial naphthoquinones (Raj et al., 2011). This indicates that *Nepenthes* pitchers do

have a chemical signaling process to detect prey that may be triggered by individual prey components.

Yilamujiang et al. (2016) performed a similar study with chitin injections. From their research, they propose a chemical signaling hierarchy: indication of the insect by chitin, then endogenous signaling via jasmonates, digestive gene expression, and finally the production of proteins. This indicates that chitin is only one of many chemical signals relevant to the induction of digestion. The authors note that this is a relatively slow process as a whole but reported that enzymatic induction by chitin is fast and fleeting. They found that the increase in proteolytic activity in response to chitin treatments occurred during a period of 24-48 hours, and that live prey was more effective at inducing chitinases than chitin alone. Another study posits that pure chitin is inferior to protein and ammonium when it comes to inducing digestive enzymes (Saganová et al., 2018). They attribute this to chitin's relative lack of nitrogen, and they suggest that proteins are universal inductors of enzymes within the pitchers of *Nepenthes*. They did, however, find that chitin induces the highest amount of a class III chitinase (compared to protein, ammonium, and whole prey) after the first 18 hours.

The goal of the present study is to determine whether the introduction of a prey stimulus will upregulate chitinase expression and to identify specific chitinases that are produced by *Nepenthes ventricosa*. We utilized RT-qPCR to measure the expression of enzymes in response to treatments of bovine serum albumin (BSA) or colloidal chitin and obtained the full-length sequence of an acidic chitinase (NrChit1) through a cloning reaction. We hypothesized that pitchers treated with chitin or BSA would exhibit increased chitinase gene expression, but our results were inconclusive.

Methods

Cloning NrChit1

Polymerase Chain Reaction (PCR)

We performed PCR using 1:20 dilutions of cDNA extracted from a mature *Nepenthes ventricosa* pitcher. Each reaction consisted of 1X PCR Gold Buffer (15 mM Tris-HCl pH 8, 50 mM KCl) (Invitrogen), 2 mM $MgCl_2$, 0.8 mM dNTP mix (New England Bio Labs), 0.025 U/µL Amplitaq Gold Polymerase (Invitrogen), 0.5 mM each primer, and 1 µL of the diluted cDNA sample (Table 1). The PCR products underwent electrophoresis at 120 V on a 1% agarose gel (1X TAE, 0.3 µg/mL ethidium bromide) until the dye neared the bottom of the gel. We utilized a 1kb ladder (Promega) for reference.

PCR Purification

In order to purify the PCR product, we used a PCR Clean-up kit (Promega: Wizard SV Gel and PCR Clean-Up System A9285). We added an equal volume of Membrane Binding Solution to each PCR product and allowed them to incubate at room temperature for 1 minute. We centrifuged the sample at 14,000 rpm for 1 minute through a SV Minicolumn. Then, we added 700 µL of Membrane Wash Solution and centrifuged the SV Minicolumn Assembly at 14,000 rpm for 1 minute. We washed the Minicolumn a second time with 500 µL of Membrane Wash Solution at 14,000 rpm for 5 minutes. We spun the sample again at 14,000 rpm for 1 minute, before eluting the DNA with nuclease free water via a 1 minute incubation and a 1 minute spin at 14,000 rpm.

Cloning

We cloned the amplicon from the PCR reaction using the NrCHIT1 primers via the pCR4-TOPO vector system (Invitrogen). Before the cloning reaction, terminal adenine residues were added to the 3' end of the product. This was achieved by adding 1 unit of Amplitaq Gold polymerase to the PCR product that was then thermocycled under the following parameters: 95 ^{o}C (1 minute), 72 ^{o}C (8 minutes), Hold at 4 ^{o}C. We transformed the vector cloning reaction into One-Shot Competent Cells that were incubated on ice for 30 minutes before applying heat shock at 42 ^{o}C for 30 seconds. We added room temperature SOC media to the transformed cells. These cells were shaken (200 rpm) at 37 ^{o}C for 1 hour, plated on a pre-warmed LB-Ampicillin plate (200 µg/ml), and incubated overnight at 37 ^{o}C.

Plasmid Mini-Prep

We inoculated colonies grown from the vector cloning reaction into TB media (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol) and incubated them overnight at 37°C. We centrifuged the subsequent bacterial cultures for 2 minutes at 6000 rpm to pellet the cells. We resuspended the bacterial cell pellet in Solution P1 (50 mM Tris, 10 mM EDTA, pH 7.5, 10 µg/mL RNase). We lysed the cells with Solution P2 (0.2M NaOH; 1% SDS) and neutralized with Solution P3 (3M potassium acetate pH 5). We centrifuged the reaction at 14,000 rpm for 10 minutes. The subsequent pellet was washed first with isopropanol and then with 70% ethanol. We dried the pellet, resuspended it in 10mM Tris (pH 8.5), and stored it at 20°C.

Measuring Chitinase Expression

Pitcher Treatments

We treated closed *Nepenthes Ventricosa* pitchers in sets of three. These pitchers were grown in a greenhouse in central Florida and treatments occurred during the fall. For each set of pitchers, we injected one with sterile colloidal chitin (1 mg/mL), one with sterile BSA (1 mg/mL), and one with sterile water (1 mL). We encased the pitchers in nylon to prevent insects from entering the pitchers, if they were to open during the treatment period. After 5 days, we harvested the pitchers and froze tissue from the bottom third at -80 ^{o}C.

Total Cellular RNA (tcRNA) Extraction

We froze the pitcher samples (1 g) with liquid nitrogen and ground them into a fine powder with a mortar and pestle. We thoroughly homogenized each sample with Plant RNA Reagent (Invitrogen) (5 mL/g of tissue), before vortexing and incubating them at room temperature for 5 minutes. In order to remove debris, we centrifuged the samples at 3,500 rpm for 5 minutes and then filtered them through sterile nylon mesh (100 µm pores). For every 10 mL of supernatant, we added 2 mL of 5M NaCl, followed by 6 mL of chloroform per 10 mL of supernatant. We centrifuged this mixture at 3,500 rpm for 30 minutes at 4 ^{o}C. We removed the aqueous phase, combining it with 0.9 volumes of Isopropanol before centrifuging the samples at 3,500 rpm for 30 minutes at 4 ^{o}C. We removed the supernatant again, combined it with 5 mL of 75% ethanol, and centrifuged it for 5 minutes at 3,500 rpm at 4 ^{o}C. We poured off the excess ethanol and left the pellet to dry for 20 minutes. We dissolved the pellet in 100 µL DEPC water. To remove any trace amounts of DNA, we treated the extracted tcRNA with DNase I. We

combined each sample with 0.1 Volume of 10X DNase I buffer and 1 µL of DNase I. We incubated this reaction at 37 ^{o}C for 15-30 minutes before adding 0.1 volume DNase Inactivation Reagent. We incubated the samples at room temperature for 2 minutes and then centrifuged them at 10,000 xg for 2 minutes. The now DNase I treated tcRNA was stored at -80 ^{o}C.

RNA Test Gel

We prepared a 1% agarose gel with 1X TAE, 1 mL of Clorox bleach, and 5 µL EtBr (10 mg/mL). We combined the samples with 5X Standard DNA sample buffer, heated them at 65 ^{o}C for 5 minutes, and then placed them on ice for 1 minute, before loading them into the gel. We ran the gel at 100 V until the dye visibly entered the gel, then running the gel at 150 V until the dye was two thirds of the way through the gel.

Complementary DNA (cDNA) Preparation

We converted the tcRNA extracted from the treated pitchers into cDNA via the First Strand cDNA Synthesis kit (Invitrogen). Up to 5 µg of tcRNA was combined with 1 µL of 50 µM Oligo(dT) and 1 µL of 10 mM dNTP mix (final volume of 10 µL). We incubated this at 65°C for 5 minutes, then placed it on ice for 1 minute. We combined the reactions with cDNA Synthesis Mix (1X RT Buffer, 11 mM MgCl2, 0.02 M DTT, and 4.5 U/µL RNaseOUT) and incubated them for 2 minutes at 42°C. We terminated the reactions by incubating them at 70°C for 15 minutes, before putting them on ice. We treated each sample with Rnase H and incubated each for 20 minutes at 37°C. We store the cDNA product at -20°C.

Real Time Quantitative PCR (RT-qPCR)

In order to measure the relative gene expression induced by the pitcher treatments, we performed a RT-qPCR using the Step One Plus RT-PCR System. We performed the reactions Rin triplicates and each individual reaction contained 50% SYBR Green Master Mix (Applied Biosystems) 0.2% of each primer and 5% cDNA. We used ribosomal RNA (18S) as an endogenous control (Table 1).

	Primers	Sequence	Cycling Parameters
PCR	NaCHIT3 F	ACA GCC ACC GCC ACA AGC ATC AAA CCA	94°C - 4 min
	NaCHIT3 R	AAG AAT AAA ATG CTA TGA CCT TAG TCA	35 x: 94°C - 30 s
	NkChitIV DGF	TTI GGI CAR ACI WSI CAY GAR AC	52°C - 30 s
	NkChitIV DGR	GAK ICC ICC RTT IAT IAT RTT NGT	72°C - 1 min
	NrChit1 F	ATG AAG ACC CAT TAT TCA TCA GCA ATT C	72°C - 4 min
	NrChit1 R	TTA AAC ACT ATC CTT GAT AGC TGA G	4°C Hold
RT-qPCR	RT NEP1 F1	CCA ACT CTG TCA AGC CCT TC	95°C - 10 min
	RT NEP1 R1	CCG AAT GTG ATA TTA GGG ATG G	40 x: 95°C - 15 sec
	RT ACTIN SAG F1	CTC TTA ACC CCA AAG CAA ACA GG	60°C - 1 min
	RT ACTIN SAG R1	GTG AGA GAA CAG CCT GGA TG	95°C - 15 sec
	RT 18S SAG F1	CTT GAT TCT ATG GGT GGT GGT G	60°C - 1 min
	RT 18S SAG R1	GTTAGC AGG CTG AGG TCT C	95°C - 15 sec

Table 1: The primers and cycling parameters used in the PCR and RT-qPCR reactions.

Results and Discussion

Identification and Cloning of a Chitinase

In order to identify chitinases from the pitchers of *Nepenthes ventricosa*, we performed PCR reactions using the primers the primers NaCHIT3 F/R (Hatano and Hamada, 2012), NkCHITIV DGF/DGR (Eilenberg et al., 2006), and NrCHIT1 F/R (Rottloff et al., 2011). Amplification was successful for NaCHIT3 and NrCHIT1, with bands appearing at around 1000 bp (Figure 3). No visible band occurred for the reaction using the NkCHITIV primers.

We cloned and transformed the successfully amplified genes into One-Shot competent *E. coli* cells via a pCR4-TOPO vector. From overnight cultures we extracted plasmids using a miniprep protocol. These plasmids were tested for proper insert size via PCR, and we observed an appropriately sized band (1000bp) for the NrCHIT1 gene (Figure 4). We sent the product of the NrCHIT1 miniprep for sequencing and confirmed the identity of the gene as NrCHIT1 (Figure 5). The stop codon present at position 43 in the translation is most likely due to a misread, or a PCR mistake (Figure 6). Future studies should be advised to focus on successfully cloning NaCHIT3, perhaps employing newly designed primers.

Figure 3: NaCHIT3 and NrCHIIT1 were successfully amplified via PCR. We performed a PCR on cDNA from *Nepenthes ventricosa* pitcher tissue using the primers NaCHIT3 F/R (A), NkCHITIV DGF/DGR (B), and NrCHIT1 F/R (C). We used the CYSP 1/5 primers as a positive control (D).

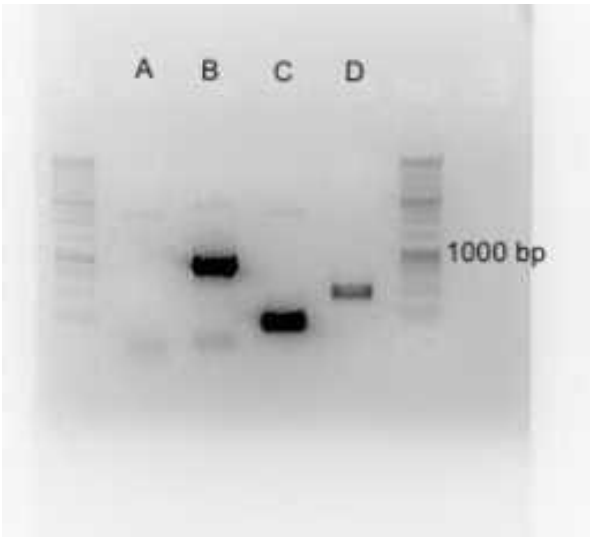

Figure 4: The potentially cloned NrCHIT1 exhibits an appropriately sized amplicon. We utilized a vector cloning reaction on potential chitinase genes that were isolated from the pitcher tissue of *Nepenthes ventricosa.* We performed a PCR on the clones with the primers NrCHIT1 F/R (A and B) and NaCHIT3 F/R (C and D). The chitinase genes should be approximately 1000 bps as seen in lane B. The corresponding clone to lane B was confirmed to be NrCHIT1.

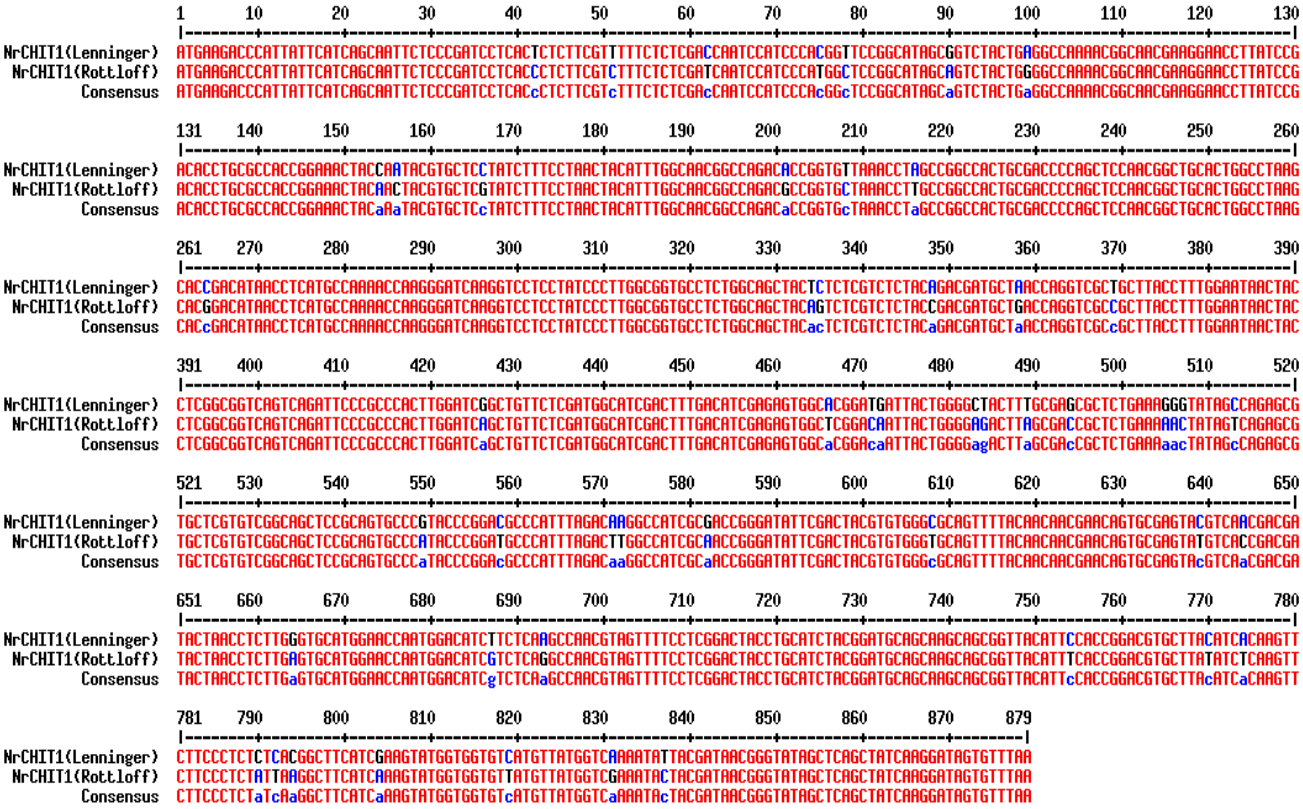

Figure 5: NrCHIT1 was successfully cloned. This is the alignment of the gene sequences of the cloned NrCHIT1 (Lenninger) to the previously identified NrCHIT1 (Rottloff).

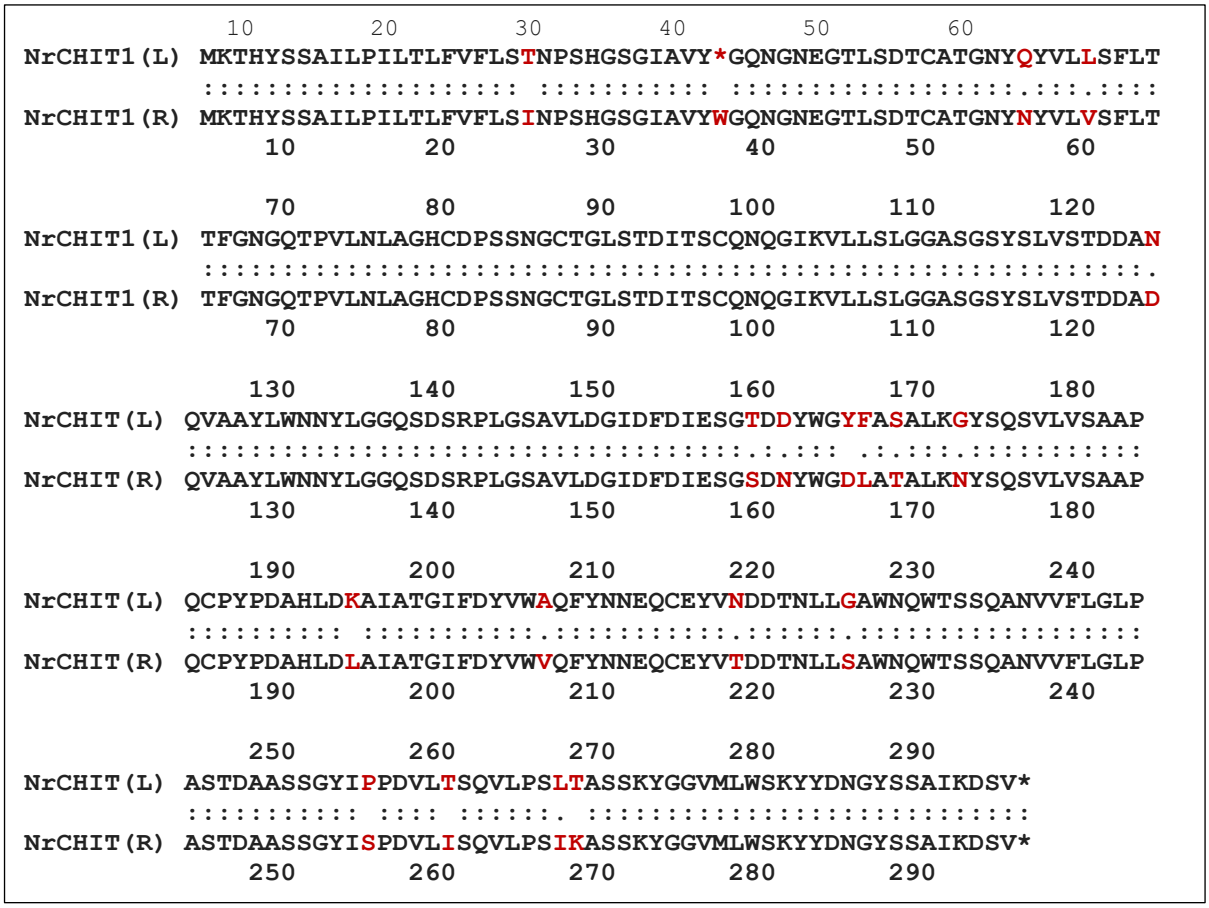

Figure 6: NrCHIT1 was successfully cloned. Here we show the alignment of the protein translations of the cloned NrCHIT1 (L) to the previously identified NrCHIT1 (R).

Analysis of Enzymatic Expression

We utilized RT-qPCR to measure the relative expression of a chitinase (NrCHIT1) and an aspartic protease (Nep1) in pitchers treated with either water, BSA, or chitin. We chose 18S rRNA as the endogenous control. For our three sets of pitcher treatments, and we ran the reactions of each RT-qPCR in triplicate. In order to normalize the data produced by the RT-qPCR, we performed a $\Delta\Delta$Ct analysis (Figure 7; Figure 8). The expression of the chitinase NrCHIT1 increased significantly in the water treated pitchers, as opposed to the pitchers

treated with BSA or Chitin (Water: 5.5x; BSA 0.1x; Chitin: 0.5x) (Figure 7). The expression of the aspartic protease Nep1 increased slightly in the BSA treatment when compared to the water treatment (Water: 3.6x; BSA: 4.1x), however this is not significant enough to draw any definitive conclusions (Figure 8).

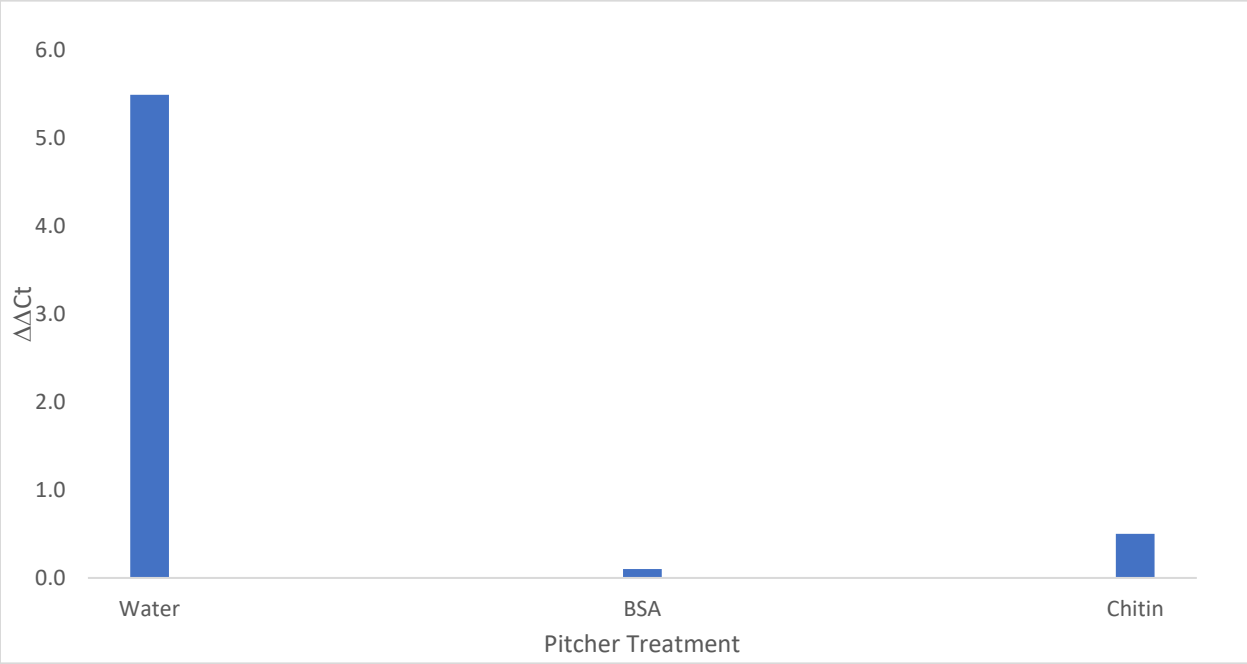

Figure 7: NrCHIT1 shows increased expression in pitchers treated with water. An RT-qPCR was performed on tcRNA extracted from *Nepenthes ventricosa* pitchers treated with water, BSA, or chitin. The ΔΔCt values of the reactions using NrCHIT1 primers were calculated as a representation of relative gene expression.

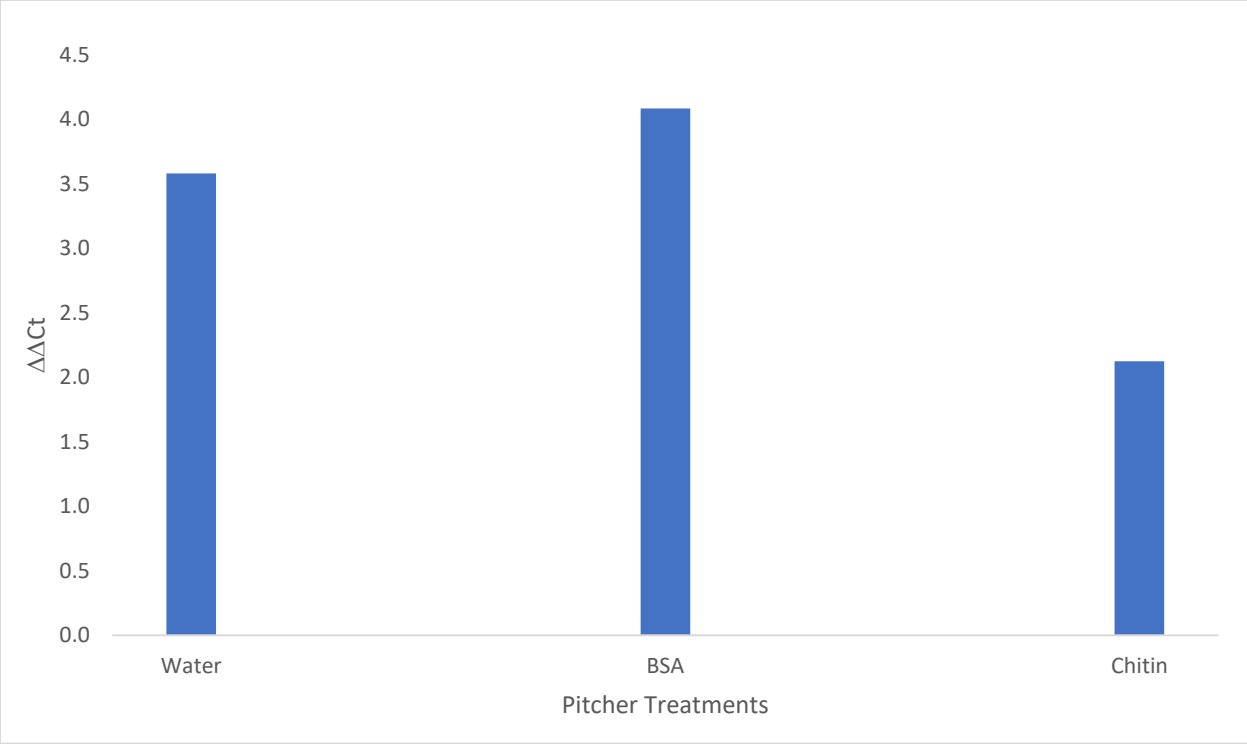

Figure 8: Nep1 shows slightly increased expression in pitchers treated with BSA. An RT-qPCR was performed on tcRNA extracted from *Nepenthes ventricosa* pitchers treated with water, BSA, or chitin. The ΔΔCt values of the reactions using Nep1 primers were calculated as a representation of relative gene expression.

The data presented here does not support our hypothesis, and further experiments are required to thoroughly evaluate the validity of our hypothesis. One possible reason for our results being inconclusive is the variability of RNA in our samples. The RNA extracted from the pitchers may have varied in concentration despite our efforts to normalize it though the relative densities of the RNA gel bands. Variability could have also been caused by disproportionate conversion of tcRNA to cDNA. It is important to note that the endogenous control (18S) also showed some variability in expression. Finding an endogenous control that is more consistent would assist in achieving more conclusive results. Future studies should also consider expanding the experiment across a broader timeline in order to assess enzymatic

expression over different spans of time. Some studies have shown that chitinase expression can be extremely variable on a day to day basis following a prey stimulus (Filyushin et al., 2019). A future RT-qPCR might also use the one-step method, instead of the two-step method utilized here, due to its increased sensitivity to certain genes and the decreased possibility for contamination (Wacker and Godard, 2005).

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