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INFLUÊNCIA DE COLÔNIAS REPRODUTIVAS DE AVES MARINHAS SOBRE POPULAÇÕES VEGETAIS NAS ILHAS SHETLANDS DO SUL, ANTÁRTICA, SOB UMA ABORDAGEM MOLECULAR

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Dedico este trabalho ao
esplendido mundo natural.

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RESUMO

A Antártica é o continente mais ao sul do globo, e também o mais gelado, onde 96% de seu território permanecem congelados durante o ano todo. Apesar de possuir as mais baixas temperaturas, altas altitudes e os mais fortes ventos, ele abriga uma grande biodiversidade. A avifauna marinha antártica é expressiva e ocupa grande parte da costa durante o período reprodutivo, compreendido no verão austral, entre os meses de outubro e março, período em que ocorre o degelo antártico. Entre pingüins, skuas, gaivotas, o *Macronectes giganteus*, popularmente conhecido como Petrel-gigante-do-sul, é uma das espécies que ocupa as áreas de degelo para a reprodução. As breeding áreas de aves marinhas, frequentemente, encontram-se associadas à comunidades e populações vegetais, dentre elas algas, liquens, musgos e plantas com flores. Os musgos *Sanionia uncinata* (Hedw.) Loeske e *Andreaea regularis* C. Muell., comuns na Antartica, junto a outras espécies compõem vastas formações verdes junto as duas únicas espécies nativas de angiospermas na região *Deschampsia antarctica* Desv. e o *Colobanthus quitensis* (Kunth) Bartl. A *D. antarctica* é uma gramínea muito comum no ambiente antártico, formando grandes gramados em diversas áreas sem ou com influência direta de colônias de aves marinhas. Essa influência ocasiona grandes depósitos de guano, porque ano após anos estas aves formam grandes colônias com dezenas, centenas ou até milhares de indivíduos. Em decorrência disso, o solo torna-se um depósito de minerais, principalmente de nitrogênio, disponível em forma de amônio e nitrato. Entretanto, nem toda a vegetação suporta essas elevadas quantidades dessas substâncias, por isso diferentes espécies de plantas evidenciam evolução nos mecanismos de tolerância ao stress por amônio, o que vêm sendo comprovado a nível molecular. Referente a isso, nos últinos anos, reguladores genéticos sensíveis à NH4+ foram identificados em *Arabidopsis thaliana* e, genes que estavam relacionados a sensibilidade à amônia, todos apresentavam respostas a nível de raiz, referenciando a absorção e concentração de amônia pelo sistema radicular das plantas. O objetivo desse trabalho foi verificar e analisar a influência das colônias reprodutivas de aves marinhas sobre as populações vegetais, nas Ilhas Shetlands do Sul, Antártica, sob uma perspectiva molecular. A partir das análises das amostras coletadas, utilizando a abordagem RNAseq e qRT-PCR foi possível identificar um único gene diferencial e significativamente expresso em *D. antarctica.* O gene

LOC_Os06g16380, dentre os tratamentos amostrados (controle, 1m e 10m), apresentou maior expressão próximos 1m das áreas reprodutivas de *M. giganteus*. O gene diferencial e significativamente expresso encontrado nesse trabalho, foi relacionado ao Heading date gene I (*Hd1*) encontrado no arroz, pois estes estão localizados na mesma região do transcriptoma. Nossos resultados sugerem que o gene LOC_Os06g16380 esteja relacionado com a capacidade da planta de tolerar altas quantidades de amônio já que, análises do solo demonstraram uma maior concentração de nitrogênio mineral disponível na forma de amônio, nas amostras mais proximas (1m) das colônias reprodutivas de aves.

Palavras-chave: Antártica; aves marinhas; *Macronectes giganteus; Deschampsia antarctica; Sanionia uncinata;* guano; nitrogênio; amônio; gene.

ABSTRACT

Antarctica is the southernmost continent of the globe, and is also the coldest one, whith 96% of its territory permanently ice-covered. Despite the lowest temperatures, high altitudes and the strongest winds, it is home to a large biodiversity. Antarctic seabirds are abundant and takes up much of the coast during the breeding season, which occurs in the austral summer period, from October to March, ice-free period in the maritime Antarctic. *Macronectes giganteus*, popularly known as South Giant Petrel, is one of the species that occupy these ice-free areas for reproduction, in addition to penguins, skuas, gulls and petrels. The breeding areas of seabirds are often associated with plant communities and populations, among them algae, lichens, mosses and flowering plants. The mosses *Sanionia uncinata* (Hedw.) Loeske and *Andreaea regularis* C. Muell., are present as vast green formations, joined by other species as the only two native species of flowering plant in the region - *Deschampsia antarctica* Desv. and *Colobanthus quitensis* (Kunth) Bartl. The *D. antarctica* is a very common hairgrass in the Antarctic environment, and is associated with breeding colonies of seabirds. These sites are large deposits of guano, because seabirds return systematically each year, forming large breeding colonies with tens, hundreds or even thousands of individuals. Due to this large supply of guano, the soil becomes a deposit of minerals, mainly of nitrogen, increasing tremendously the soil contents of ammonium and nitrate. The problem is that not all vegetation support such high quantities of these substances, so different plant species show trends in the mechanisms of tolerance to stress by ammonium, which have been proven at the molecular level. In recent years, genetic regulators sensitive to NH_4^+ were identified in *Arabidopsis thaliana* and genes that were associated with sensitiveness to ammonia all showed responses at the root level, referencing the absorption and ammonia concentration by the root system of the plants. The aim of this study was to investigate and to analyze the influence of breeding colonies of seabirds on plant populations in the South Shetland Islands, Antarctica, from a molecular perspective. From the analysis of the collected samples using the RNA-seq and qRT-PCR approach, it was possible to identify a single differential gene, which was significantly expressed in *D. antarctica*. The LOC_Os06g16380 gene among the sampled treatments (control, 1m and 10m), showed higher expression coming 1m near breeding areas of *M. giganteus*. The gene differentially

expressed in this work has been described in literature and was related to Heading date I gene (*Hd1*) found in rice, since they are located in the same region of the transcriptome. Our results suggest that LOC_Os06g16380 gene is associated with the plants ability to tolerate high amounts of ammonium, as soil analysis demonstrated larger contents of ammonium in the nearest sampling sites (1m) of breeding areas of seabirds.

Keywords: Antarctica; seabirds; *Macronectes giganteus*; *Deschampsia antarctica*; *Sanionia uncinata*; guano; nitrogen; ammonium; gene.

LISTA DE ILUSTRAÇÕES

APRESENTAÇÃO

A presente dissertação foi organizada em duas partes, iniciando com uma introdução geral do assunto e um capitulo em forma de artigo científico. A introdução geral apresenta informações sobre a vegetação antártica, especialmente as duas espécies foco do estudo *Sanionia uncinata* (Hedw.) Loeske e *Deschampsia antarctica* Desv.*,* uma breve descrição da avifauna marinha e das Ilhas e península Antarctica. Apresenta também, informações dos métodos de análise molecular aplicados às amostras da vegetação.

A segunda parte é constituída pelo capítulo 1 intitulado: **Overexpression of Head date 1 gene (*Hd1*) relationship on the adaptation of *Deschampsia antarctica* Desv. and *Sanionia uncinata* (Hedw.) Loeske to guano input from *Macronectes giganteus* colonies of Antarctica.** Apresenta uma análise da influência das áreas de reprodução de *Macronectes giganteus* sobre as populações vegetais de *Sanionia uncinata e Deschampsia antarctica,* utilizando uma abodagem de RNA seq e um qRT-PCR a posteriori. Artigo formatado nas normas da Revista Nature Plants.

SUMÁRIO

1. INTRODUÇÃO GERAL

A biodiversidade Antártica

O continente antártico, embora remoto é reconhecido como o símbolo de último grande deserto. A presença humana no Oceano Austral e no continente começou no início de 1900 com a pesca, a caça e a exploração local, e muitas espécies de plantas e animais invasores foram deliberadamente introduzidas em várias ilhas sub-antárticas (BARGAGLI, 2008).

A maioria dos recursos da Antártica são diferenciados em comparação com ambientes e biota de latitudes mais baixas e, apesar de ser cercado por mares que foram navegados por apenas 200 anos, o único continente despovoado foi explorado apenas nos últimos 100 anos (BENNINGHOFF, 1987; SHIRIHAI, 2008).

A maior parte da fauna da Antártica vive nos oceanos que circundam o continente Antártico, dentre elas são encontradas cerca de 40 espécies de aves marinhas que reproduzem em áreas descobertas de gelo formando colônias com variado número de indivíduos (BARGAGLI, 2008; CROXALL *et al.*, 2002). E devido sua ampla distribuição no ambiente, estas aves vem sendo estudadas como bioindicadoras da qualidade ambiental dos ecossistemas marinhos, e no caso de instabilidade destes, elas tendem a buscar outro ecossistema favorável a sua permanência e sobrevivência (SANDER *et al.*, 2006).

Regiões costeiras são áreas importantes para várias espécies de aves marinhas que estabelecem colônias nas Ilhas Shetland do Sul (WARREN; DEMER, 2010). Dentre essas ilhas, a Ilha Rei George e a Ilha Elefante são locais de reprodução de aves marinhas, como os pinguins-papua (*Pygoscelis papua)* e pinguins-de-barbicha *(Pygoscelis antarcticus),* skuas (*Catharacta maccormicki*e *C. antarctica*), gaivotões (*Larus dominicanus*) e petréis gigantes (*Macronectes giganteus),* quesão importantes componentes da avifauna Antártica (HARRIS *et Al.*, 2015) (Fig. 1.1e 1.2).

A ave de maior envergatura de asas da Antártica é o petrel-gigante-do-sul (*Macronectes giganteus*), que escolhe como área de reprodução grandes platôs, formando colônias de poucos a centenas de ninhos, dependendo do local (Fig.1.3). A alimentação dessas aves é diferenciada entre machos e fêmeas, onde os machos se alimentam, principalmente durante a época reprodutiva, de carniça adquirida em viagens a outras

zonas costeiras, e as fêmeas de krill, lulas e peixes capturados no mar (GONZÁLEZ-SOLÍS *et al.*, 2002; PETRY; KRUGER, 2011). Uma das fontes de alimentação do petrel-gigante-do-sul, o krill (*Euphasia superba)* é um crustáceo abundante na Antártica que serve como base da cadeia alimentar para diversos animais (CROXALL et al., 2002, PETRY, 1994). A distribuição e forrageio da maioria das aves marinhas da Antártica se sobrepõe a distribuição e concentração do krill, o que ressalta sua grande importância na cadeia trófica da Antártica (SANTORA *et al.*, 2009; FORCADA *et al.*, 2006).

No ecossistema antártico de áreas de degelo, além da fauna, a vegetação está representada por apenas três espécies de plantas com flores, sendo duas nativas, *Deschampsia antarctica* Desv. (*Poaceae*) e *Colobanthus quitensis* (Kunth) Bartl (*Caryophyllaceae*), além de *Poa annua* L. (*Poaceae*), introduzida acidentalmente na década de 1980. As briófitas senso lato que ocorrem na Antártica são reunidas em dois grandes grupos taxonômicos: as *Marchatiophyta* (hepáticas), representadas na área por 22 espécies, e as *Bryophyta* (musgos) com cerca de 110 espécies claramente reconhecidas, sendo uma delas a *Sanionia uncinata*, espécie com grande representatividade entre os musgos. Ressalta-se que muitos outros nomes já foram propostos para espécies da região, os quais caíram em desuso em virtude de erros na identificação, ou na classificação. Cabe destacar que, no ambiente terrestre, também ocorre a espécie de alga macroscópica *Prasiola crispa* (Lightfoot) Menegh. (*Clhorophyta*), a qual por ser ornitocoprófila apresenta considerável biomassa principalmente em torno das pinguineiras (PEREIRA; PUTZKE, 2013; OLECH, 1996; VICTORIA *et al.* 2009) (Fig.1.4 e 1.5).

Essas áreas que ficam descobertas de gelo entre os meses de novembro e março, são habitadas por vários animais marinhos, como focas, pinguins, e também pela vegetação, tais como musgos, líquens e algas. As colônias de animais marinhos, as comunidades vegetais e suas interações formam um importante e especial ecossistema. Entretanto, a cada ano uma grande quantidade de guano de aves marinhas é depositado nos ecossistemas terrestres antárticos, o que leva a formação de solos ornitogênicos, que são por sua vez ricos em carbono orgânico, nitrogênio e fósforo, e possuem grandes variações no pH (ZHU *et al.*, 2011).

Os locais de nidificação das aves marinhas, se tornam, por sua vez, pontos biológicos com grande produtividade devido a esse acúmulo de guano (XIAODONG *et al.*, 2013). As comunidades vegetais são amplamente afetadas pelo depósito do guano,

sendo assim, muitas populações de plantas são descritas como ornitocoprófilas e ornitocoprófobas. Espécies ornitocoprófilas são aquelas que vivem e toleram o guano das aves, e espécies ornitocoprófobas são aquelas que não toleram(tem fobia) do guano recorrente das aves, caracterizando assim quais espécies vegetais ocorrem próximos e junto às colônias reprodutivas de aves marinhas (PEREIRA *et al.*, 2010).

Segundo Victoria & Pereira (2007) *Sanionia uncinata* é uma das espécies de musgos de maior abundância nas áreas litorâneas da Antártica, ocorrendo em substratos ricos em matéria orgânica, próximos as colônias de aves, por isso chamadas de ornitocoprófilas. Além dos musgos outra espécie ornitocoprófila bastante encontrada nas áreas livres de gelo da Antártica é a *Deschampsia antarctica,* uma das duas espécies nativas de angiospermas presentes em toda costa e ilhas da Antártica (ALBERDI *et al.*, 2002; BARCIKOWSK *et al.*, 1999).

As espécies vegetais que crescem junto a colônias de aves recebem um maior aporte de amônio, nitrato e fosfato, mostrando claramente que a densidade da colônia de aves marinhas têm um papel determinantena concentração de nutrientes no solo e da composição vegetal que cresce no local (ELLIS *et al*. 2006). Em ilhas do Golfo da Califórnia também foi evidenciado que, a deposição de amônio nos solos adjacentes às colônias de aves marinhas, altera a concentração de nutrientes e a biomassa de plantas e de detritos, aumentado a produção primária, o que gera um grande impacto no desenvolvimentodas plantas, aumentando a densidade e a extensão dos tapetes de musgos e de gramíneas (ANDERSON & POLIS 1999), entretanto, quando o amônio é a única fonte de Nitrogênio da planta ele pode ser tóxico (GERENDÁS *et al.*, 1997).

Como as plantas necessitam de nitrogênio para seu crescimento e desenvolvimento, as colônias de aves marinhas, muitas vezes, acabam sendo a principal fonte deste nutriente, grande parte adquirido na forma de amônio (NH_4)(BRITTO, 2002). Nesse sentido, diferentes espécies de plantas evidenciam evolução nos mecanismos de tolerância ao stress por amônio, o que vêm sendo comprovado a nível molecular. Recentemente reguladores genéticos sensíveis à NH4+ foram identificados em *Arabidopsis thaliana* e, entre eles, o WT, GMPase e seus mutantes apresentaram mudanças na sobrevivência e atividades celulares a nível de raiz (QIN *et al*, 2008). Li *et al*. (2012), em seus estudos com *Arabidopsis thaliana,* analisou os genes que estavam relacionados a sensibilidade à amônia, todos apresentando respostas a nível de raiz, referenciando a absorção e concentração de amônia pelo sistema radicular das plantas.

A abordagem RNA-Seq

O RNA-seq é uma abordagem desenvolvida para inferir e quantificar transcriptomas utilizando tecnologia de deep-sequencing. O RNA, composto por mRNAs, RNAs não codificantes e pequenos RNAs, é convertido em uma biblioteca de cDNA composta por fragmentos com adaptadores atrelados. Os fragmentos então são sequenciados gerando "reads" de cerca de 30 a 400 pares de base cada (Wang,Z., Gerstein, M. & Snyder, M., 2009) .

qRT-PCR

Real-time quantitative reverse-transcription polymerase-chain-reaction (qRT-PCR) é um método utilizado para quantificar a expressão de genes mRNA, e é considerado o método mais sensível para detectar ácidos nucleicos, além de ser uma tecnica rápida e de alto rendimento (BAR *et al.* 2003; DALLAS *et al.* 2005; FLEIGE; PFAFFL, 2006;).

Assim, o objetivo deste trabalho é avaliar a influência das colônias de *Macronectes giganteus* sobre as populações de *Sanionia uncinata* e *Deschampsia antarctica* na Antártica, utilizando a análise transcriptomica dessas plantas. Afim de testar a hipótese do porquê, espécies vegetais crescem junto a áreas de reprodução de aves e, que apresentam uma expressão diferencial de genes relacionados ao transporte de nitratos do que aquelas que crescem em locais sem a influênciadas aves marinhas da Antártica.

Assim, a questão biológica envolvendo a influência das colônias de aves sob a adaptação de plantas ao ambiente antártico, ainda não avaliada sob o ponto de vista da biologia molecular, basea-se nas seguintes perguntas: quais genes são diferencialmente expressos quando essas plantas estão expostas a áreas onde aporte de nitrogénio é alto, como o guano? A presente proposta apresenta carácter inovador, uma vez que estratégias de análise de transcritos, como o caso do RNA-Seq ainda não foram exploradas para avaliação dos processos adaptativos relacionados as interações entre as aves e as plantas da Antártica.

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2. CAPÍTULO I

Overexpression of Head date 1 gene (*Hd1*) relationship on the adaptation of *Deschampsia antarctica* Desv. and *Sanionia uncinata* (Hedw.) Loeske to guano input from *Macronectes giganteus* colonies of Antarctica.

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Overexpression of Head date 1 gene (*Hd1*): an adaptation of antarctic hairgrass to guano input from *Macronectes giganteus* colonies of Antarctica.

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Abstract

The Antarctic biodiversity, beyond the species composition, also comprises interactions between fauna and flora. *Macronectes giganteus*, is one of the species that occupy the antarctic ice-free areas for reproduction. The moss *Sanionia uncinata* (Hedw.) Loeske and *Deschampsia antarctica* Desv., common in Antarctica, with other species make up vast green formations and is associated with breeding areas of seabirds. These sites are large deposits of guano, because a large amount of birds those gather in colonies. Due to this large supply of guano, the soil becomes a deposit of minerals, mainly of nitrogen available in the form of ammonium and nitrate. The problem is that not all plant species tolerate high quantities of these substances so different plant species show trends in the mechanisms of tolerance to stress by ammonium, which have been proven at the molecular level. The aim of this study was to investigate the influence of breeding colonies of seabirds on plant populations in the South Shetland Islands, Antarctica, from a molecular perspective. From the analysis of the collected samples using the RNA-Seq and qRT-PCR approach was possible to identify a single gene differential and significantly expressed in *D. antarctica*. The LOC_Os06g16380 gene among the sampled treatments (control, 1m and 10m), showed higher expression coming 1m near breeding áreas of M. giganteus. Our results suggest that Hd1 is associated with the plants stress related to guano input since that soil analysis demonstrated a higher concentration of mineral nitrogen available near of breeding areas of seabirds.

Significance statement

The antartic hair grass *Deschampsia antarctica* Desv. shows an differential gene expression when submitted to an increased guano input on soil close to seabirds breeding areas. An Head date 1 (Hd1) homologue was the single gene differentially expressed in

the Antarctic hair grass roots profile. This gene locus was reported as a controlling response to photoperiod, however the present data suggest that may be related with the nitrogen homeostasis in grass species.

Introduction

The Antarctic terrestrial ecosystems are characterized by extreme abiotic conditions when compared with other continents. Around 86% of Antarctic is covered by ice, the air temperatures are low (average between the -10 ° C to -20 ° C in coastal areas) and high winds and snowstorms cover the region which also presents a short summer season (about two months). Altogether those features hinder the establishment of terrestrial biodiversity. Besides the environmental constraints the animals' influence during the short austral summer, increase the accumulation of large quantities of minerals in the soil (mainly Nitrogen) during the breeding season (Alberdi *et al.*, 2002; Lee *et al.*, 2008; Barcikowski *et al.*, 2001).

Deschampsia antarctica Desv. (Figure 1a) is one of the two native flowering plant species found in Antarctica and is the only hairgrass inhabiting the region. This species is abundant mainly in the South Shetlands Islands occupying almost the entire coastal area of the Maritime Antarctic, often occurring in the vicinity of bird colonies (Edwards and Lewis-Smith *et al.*, 1988; Parnikoza *et al*., 2011).

Moreover, mosses are important representatives of the Antarctic terrestrial ecosystems, often composing the main landscape of coastal areas and forming large Green carpets of vegetation. *Sanionia uncinata* (Hedw.) Loeske (Figure 1b) is one of the most abundant species of mosses in Polar Regions and contributes to the accumulation of organic matter in the Antarctic soils (Mendonça *et al.*, 2011; Neufeld *et al.*, 2015). This moss species occurs mainly in environments with constant water supply, like those close to the drain lines coming from defrosted water (Tojo *et al.*, 2012; Lud *et al.*, 2002).

Considering that the spatial distribution of vegetation in Antarctica is closely linked to marine animals, understanding how changes in the seabird communities may affect terrestrial communities becomes necessary to determine the degree of interaction between plants and seabirds (Barcikowski, 2001). It is expected that one of the key elements is the input of nitrogenous compounds originating from the excrement of seabirds that may influence both positively and negatively the plant communities. Increases in ammonium input in studies of soil composition influenced by seabirds were

reported in several analysis of soil composition in areas under the influence of marine animals, such as Santos *et al*. (2006), Park *et al*. (2007), Sun *et al*. (2002), Theobald *et al*. (2013).

In the nitrogen cycle, ammonium is replaced by an important role in living organisms that receive the nitrogen supply to its basic functions, and seabirds are responsible for large amounts of this issue (Zhu *et al*., 2011). This especially occurs in the Antarctic coastal regions and sub Antarctic places where the ocean has great productivity resulting from large numbers of seabirds breeding over Antarctica every year (Riddick *et al*., 2012).

The main nitrogen source come from the energy flow from the fish- and crustaceans-based diet of penguins, petrels and gulls (Petry *et al*., 2008, 2010; Copello *et al*., 2008; Hebert *et al*., 2009). Although the average NH_4^+ concentrations of soils are often 10–1000 times lower than those of NO_3^- (Marchner, 1995), the difference in soil concentrations does not necessarily reflect the uptake ratio of each N source. Indeed, the role of NH_4^+ in plant nutrition has probably been under estimated, because most plants preferentially take up NH_4^+ when both forms are present. Ammonium requires less energy for uptake and assimilation than nitrate, mainly because NO_3^- has to be reduced prior to assimilation (Bloom *et al*., 1992). Optimal plant growth is, however, usually achieved when N is supplied in both forms (Bloom *et al*., 1999). On the other hand, the excess of ammonium in soils, may adversely affect the growth, productivity, tolerance to drought or frost and resistance to diseases and insects, leading to long-term changes in species composition. The excess of ammonium also causes soil acidification and eutrophication (Fangmeier *et al*., 1994; Wilson *et al.*, 2004), changing the atmospheric composition and land nutrient supply (Blackall *et al.*, 2007).

Abiotic stress caused by cold, drought and increased salinity (e.g. excess of ammonium), generated a selective pressure for plants to develop mechanisms that would enable their development in environments where these factors reach extremes. This adaptive process resulted in a gene pool facing to a successful survival strategy for climate change, especially those of unexpected and extreme level (Lee et al., 2013). Lee et al. (2008), using the Expressed Sequences Tag (EST) approach, generated by large scale single-pass sequencing of cDNA clones, found novel genes of *D. antarctica*, related to the differential response of the species abiotic stress in Antarctic environment,

demonstrating the strong selective pressure under the Antarctic plants, but the action of these genes have not experimentally clarified.

Thus, the biological question involving the breeding influence under the adaptation of plants to the Antarctic environment is: which genes (if so) are differentially expressed when those plants are exposed to the higher nitrogen compounds input, as guano? Within this work we attempted to answer this question by using the transcriptome-based analysis of two species of Antarctic plants, *Deschampsia antarctica* and *Sanionia uncinata*, both common species in the nesting areas of Southern Giant Petrel (*Macronectes giganteus*) and comparing the transcriptome of those plants with plants from the same species in areas without influence of guano.

Results

Differential expression analysis. As expected the gene expression analysis distribution across treatments were distinct for each species tested. The moss species presented lower differential expression detected within treatments (Figure 2a). Furthermore Kernel distribution of FPKM scores for overall genes detected across the Antarctic hairgrass *Deschampsia antarctica* indicated a similar distribution of transcripts within the treatments (Figure 2b), meaning that only this plant was affected by the guano from the Southern Giant Petrel. Following the kernel analysis Cufflink tool was applied in order to determine which genes were differentially expressed in *Sanionia uncinata* and *Deschampsia antarctica* were identified a single significant gene expressed and only for the grass species. The LOC_Os06g16380 gene had its expression in *D. antarctica*, and among the three treatments (control, 1m and 10m), with higher expression close to the *Macronectes giganteus* colonies (1m treatment). The moss species does not show a significant gene expressed for both treatments.

Confirmation of differentially expressed genes by qRT-PCR analysis. Having found the LOC_Os06g16380 gene differentially expressed in *D. Antarctica* by RNA-Seq, the next step was to perform a qRT-PCR on total mRNA to confirm the expression patterns. A higher and significant concentration of the target gene fragments was observed in *D. Antarctica* at 1m of distance from *M. giganteus* colonies in both sampling places (Copacabana and Stinker Point) (Figure 3). At 5m and 10m of distance from the breeding colonies the expression patterns were similar to each other but smaller than at 1m of distance (Figure 3). A higher amount of ammonium in the soil near the breeding colonies

(e.g. 1m) in both sites (Copacabana - 1.8 mg/dm^3 and Stinker Point - 1.5 mg/dm^3; Figure 4) was detected suggesting the influence of nitrogen in the differential expression of LOC_Os06g16380 gene.

Soil mineral nitrogen analysis. Through the Kjeldahl distillation of mineral nitrogen in the soil, there was obtained values nitrogen as ammonia and nitrate. There was a higher amount of ammonium in the soil near 1m colonies breeding of birds in both sites: Copacabana (1.8 mg / dm3) and Stinker Point (1.5 mg / dm3). Nitrogen in nitrate form does not significantly varied between the three samples at both locations (Figure 4).

Discussion

The LOC_Os06g16380 gene was previously found and described by Zhang et al. (2012) as belonging to a region related with the Heading date gene I (*Hd1*) found in rice. This gene is an orthologue gene of *CONSTANS* gene indentified in *Arabidopsis* model species (Takakashi & Shimamoto, 2011), and regulates the expression of florigen gene *Hd3*, responsible for controlling the mechanism involved with the transition from the vegetative to the reproductive phase in flowering plants (Kojima *et al*., 2002; Sonoda *et al*., 2003; Park *et al*., 2006). In rice, the *Hd1* gene is reported as the major quantitative trait locus (QTL) controlling response to photoperiod (Yano et al., 2000) that determines the regional and seasonal adaptation of rice crops (Zhang *et al*., 2012). This trait conferring short or long vegetative phase, susceptible to use in breeding program to increase the yield in distinct latitudes (Takakashi & Shimamoto, 2011; Zhang *et al*, 2015). However, the pleiotropic effect from *Hd1* expression on the productivity/yield and growth in rice was already observed (Zhang *et al*. 2012), although this gene did not affect these characteristics, and thus expression was not detected in roots. Our results in *Deschampsia antarctica* suggests that the higher input of NH_4^+ close to the seabirds colonies induced an increase in the LOC_Os06g16380 expression and that this region can be related with the capacity of this grass species to respond to high contents of ammonium in soil and even be related to the transport of these mineral in grass roots. These findings corroborate the theory of guano input from sea mammals and birds enables in which nutrients can change the chemical and organic characteristics of the soil and in turn can determine the spatial distribution of *D. antarctica* (Smykla *et al*, 2007;Park *et al*, 2012).

The absence of root in mosses would be a suitable explanation for non-significant result of differential gene expression in *Sanionia uncinata*. Instead of roots, mosses

possess rhizoids that are not the main routes for uptaking water and nutrients. Otherwise angiosperms have developed roots to perform this function (Raven & Edwards, 2001; Victor & Dolan, 2012).

Regarding *Sanionia uncinata*, no significant results relating to differential gene expression in the 3 treatments suggest that this is not an ideal moss to this type of analysis. This is probably due to their morphological plasticity associated with its great ecological amplitude (Gimingham & Smith, 1971; Hebel *et al.,* 2012.), being the mechanisms that control these adaptations remains unknown up to date.

Methods

Plant materials. Pant samples were colected in Copacabana, near the US Refuge in King George Island (62° 23'0 "S, 58° 27' 0"W) and, in Stinker Point, Elephant Island (61° 13' 20" S, 55° 21'35" W), during the Antarctic austral summer of 2014/2015. *Deschampsia antarctica* and *Sanionia uncinata* were sampled at pre-defined locations according to the breeding areas of *Macronectes giganteus* (Southern Giant Petrel). Plants were collected from approximately 1m, 5m and 10m from the center of the breeding area, with three replicates per sample, totalizing 9 samples per plant. The aerial part, and roots in the case of *D. Antarctica,* were removed from soil and placed in zip-loc bags. Four different individuals were taken along each sampling distance. The same procedure was applied to collect plant samples in an area without influence of bird colonies. Subsequently the samples were storage at -80°C until RNA extraction.

RNA Extraction. Specimens collected in the field were washed with autoclaved ultrapure water, and homogenized with liquid nitrogen, crucible and pestle. The homogenate obtained for each sample were used for total RNA extraction. RNA extraction was performed for each treatment, three replicates (n = 18) using the connector kit of pure RNA Mini kit ® (Ambion Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The quantity and quality of total RNA was measured by spectrophotometry using a Spectrophotometer NanoVue ™ Plus (GE Healthcare, Little Chalfont, United Kingdom).

mRNA Enrichment. Total RNA were subjected to enzymatic digestion of DNA using the Kit™ TURBO DNA-free (Ambion Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The cytoplasmic depletion (5S, 5.8S, 18S, and 28S) and mitochondrial (12S and 16S) ribosomal RNA (rRNA) was performed with

the Eukaryote v2 RiboMinus ™ System (Ambion Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions, and mRNA was quantified by fluorometry using a Qubit - RNA Assay Kit (Invitrogen, Carlsbad, California, USA).

Library preparation and sequencing. Eighteen libraries (one for each treatment for both species) were generated using Ion Total RNA-Seq Kit v2 (Ambion Life Technologies, Carlsbad, California, USA). Ion OneTouch ™ 2 System and Ion PGM ™ Template OT2 400 Template Kit were used to prepare RNA library, sequencing was performed using the Ion PGM ™ Sequencing 400 in Ion system PGM ™ using three Ion 318 ™ Chip v2 (six loaded samples per chip).

Assembly and mapping transcripts. The quality filtering of RNA-seq reads for each library were independently mapped using TopHat2 against the *Physcomitrella patens* v3.0 and *Oryza sativa* v3.0 as reference genomes for *Sanionia uncinata* e *Deschampsia antarctica,* respectively. The levels of gene expression and isoforms were first normalized using a variation of the FPKM method (Fragments Per Kilo-base of mRNA length per Million mapped reads) performing in the Cufflinks tools (Cufflinks2, CuffMerge and Cuffdiff2) on the alignments of TopHat, and *P. patens* genome annotation v3.1 and v3.1 *O. sativa*. All analysis was performed in the Galaxy, the Galaxy Rätsch Lab platform (galaxy.cbio.mskcc.org/). The genomes and *P. patens* and *O. sativa* annotation of genes were downloaded from Phytozome V10.1 (phytozome.jgi.doe.gov). Statistics and graphical analysis of the differential expression on the transcripts detected were performed in the program R (version 3.1.1) with CummeRbund extension (Goff et al., 2013).

qRT-PCR analysis. The candidate gene selected from the RNA Sequence analysis had the expression patterns analyzed by qRT-PCR using SYBR® Green detection system (Applied Biosystems®, California, USA). The quantitative variation between different samples was evaluated using the comparative CT method (ΔΔCT), and the data of target gene expression, normalized to the level of expression of TIP41-like genes, used as standard reference in internal control (endogenous) (Caldana, et al., 2007, Jain et al., 2006). The qRT-PCR reactions were performed in triplicate techniques from: 2.0 ul 10x buffer; 1.2 uL of 50 mM MgCl2; 0.4 uL of 5 mM dNTPs; 1 ul of each oligonucleotide (10 mM); 0.05 uL Taq Platinum - DNA polymerase (5 U / uL); 2 uL of Syber Green (1x); 0.4 uL ROX, the first tape 1 ul cDNA (diluted 1: 5) and water to make a final volume of 20 uL. The cycling conditions used for amplification were 50 °C for 2 minutes, 95 °C for

10 minutes, and 40 cycles of 95 ° C for 30 seconds, 60 °C for 1 minute and 72 °C for 1 minute, occurring reading fluorescence in this last step. Finally, a cycle of 72 °C for 5 minutes.

Soil mineral nitrogen analysis. In each GHG sampling event, sSoil samples were taken for analysis of mineral nitrogen (nitrate – NO_3 and ammonium – NH_4 by Kjeldahl distillation), determined according to Tedesco et al. (1995).

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Contributions

C.K.P carried out experiments in *Sanionia uncinata* and *Deschampsia antarctica*, carried out the RNA extraction for RNA-Seq analysis, perfomed the initial transcriptome assembly and wrote the manuscript with assistance from the co-authors. A.D.M.B carried out the extraction, purification and normalization of RNA for RNA-Seq analysis. R.P.A. carried out the soil samples from antarctic plants rhizophere and the mineral nitrogen content analysis. L.F.W.R. designed and carried out the RNA-Seq sequencing. A.B.P. carried out the plantscollections in Antarctica. M. V. P. carried out the plant collections in Antarctica, designed and co-directed the project. F.C.V. conceived and co-directed the Project, designed and carried out the bioinformatic analysis and transcriptome assembly.

Conflict of Interest

The authors declare no competing financial interests.

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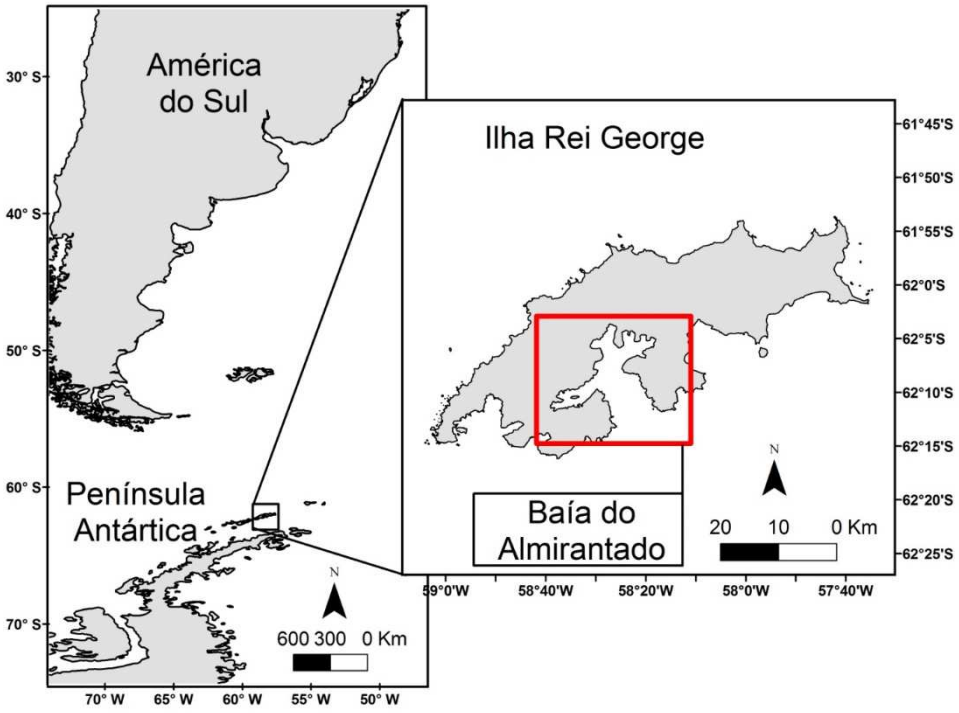


Figura 1.1 Localização das áreas de estudo, Ilha Rei George, Ilhas Shetlands do Sul, Antártica.

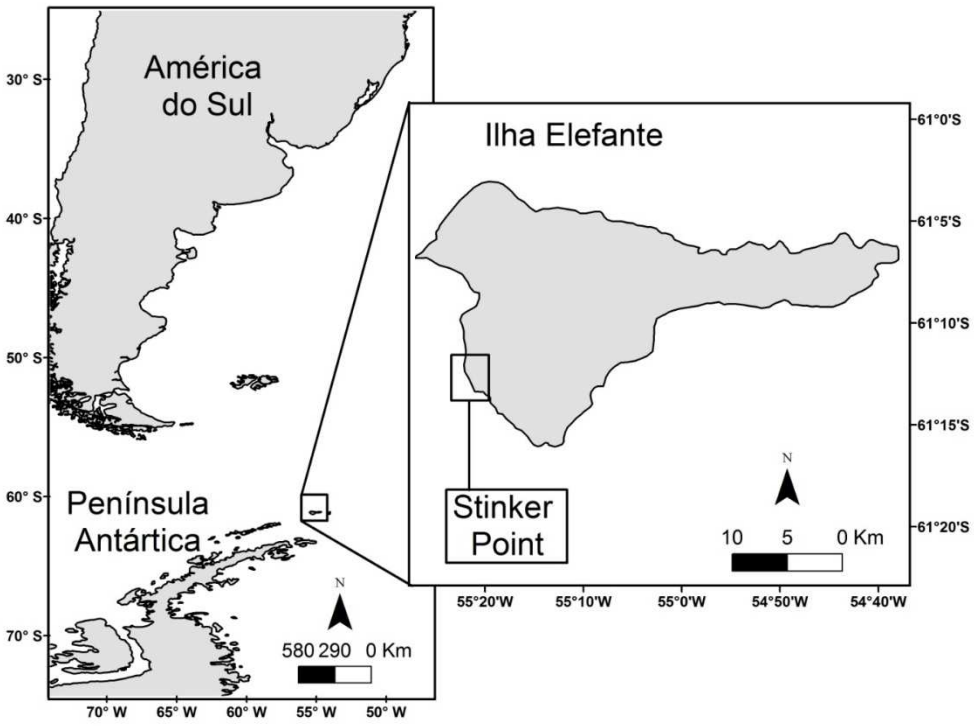


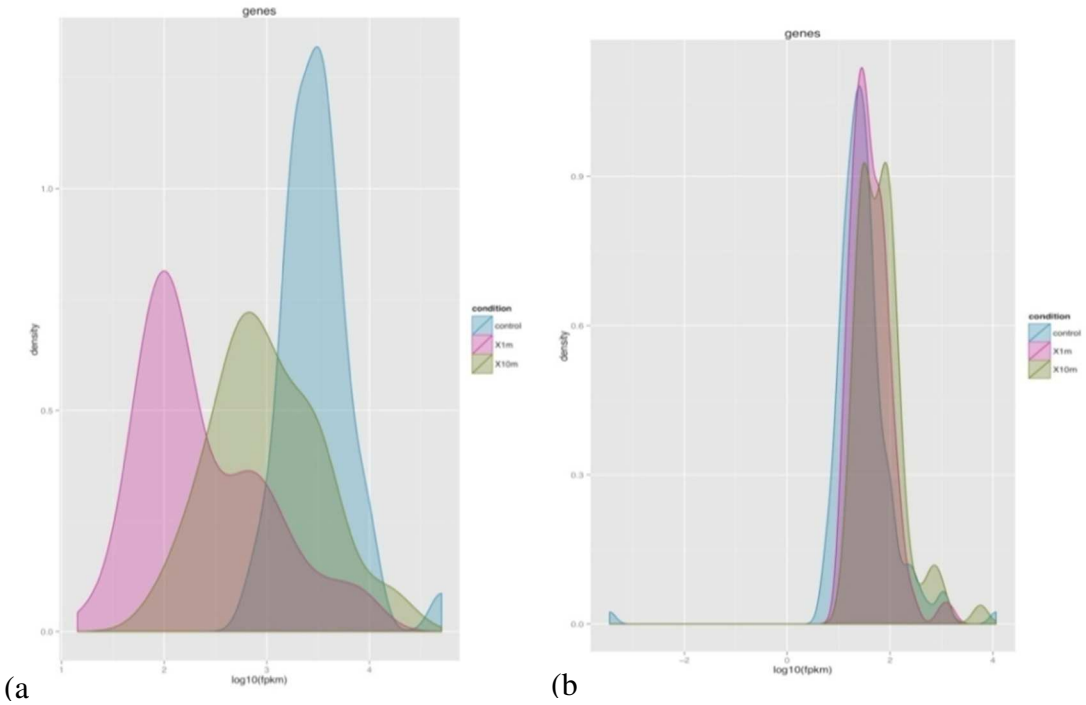
Figura 1.2 Localização das áreas de estudo, Ilha Elefante, Ilhas Shetlands do Sul, Antártica.

Figura 1.3 Southern Giant Petrel: *Macronectes giganteus*.

Figura 1.4 Formação vegetal em carpete: com predominância de *Sanionia uncinata.*

(a) (b)

Figure 1.5 Selected plant species for the study. (a) Antartic Hairgrass *Deschampsia Antarctica* Desv. (Photo: Clarissa Kappel Pereira). (b) *Sanionia uncinata* (Hedw.) Loeske, the most common moss carpet for antarctic ice-free areas (Photo: Filipe de Carvalho Victoria).



(a (b

Figure 2.1 Fragemnts per kilobase of exon per million reads mapped (FPKM) scores for overall genes detected in RNA-Seq experiments. (a) Lower distribuition on trancrripts of *Sanionia uncinata* detected across treatments, otherwise displayed for *Deschampsia antarctica* (b), suggesting the influence of the treatments only for the Antarctic hairgrass.

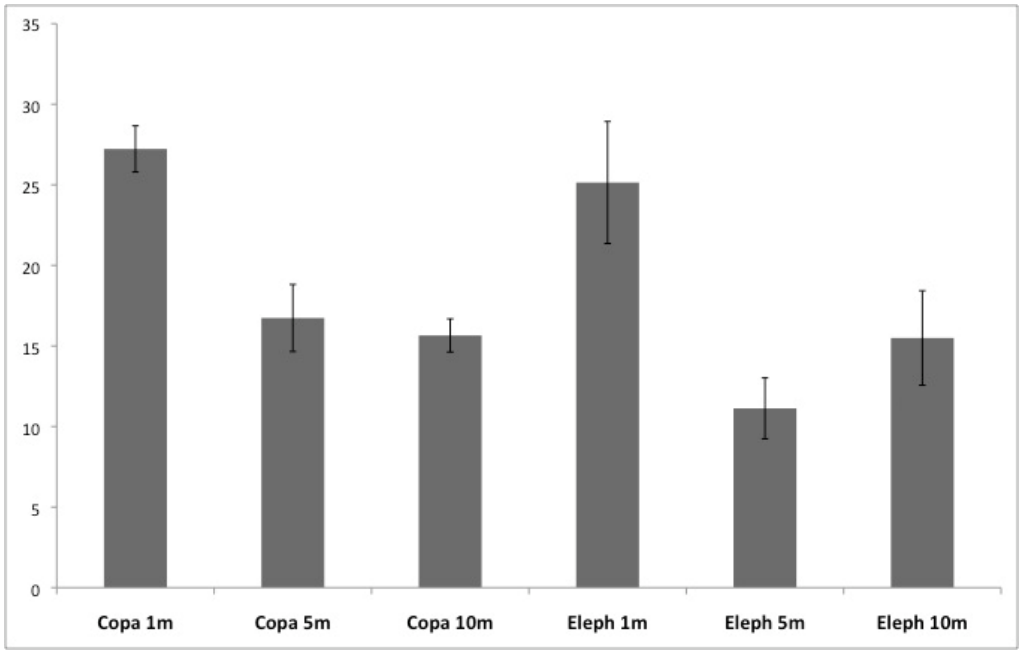


Figure 2.2. Fragments quantities of LOC_Os06g16380 gene in the three treatments of *Deschampsia antarctica in* 1m, 5m and 10m, sampled in Copacabana (Rey George Island) e Stinker Point (Elephant Island).(Teste estatístico de variância)

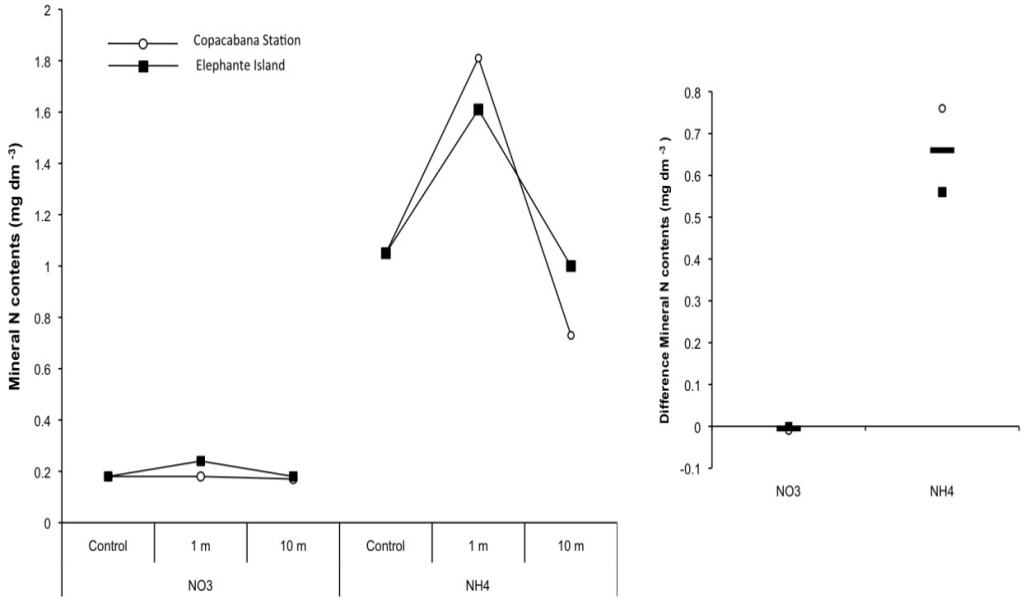


Figure 2.3 Mineral Nitrogen concentrations in soil of *Deschampsia antarctica in* control treatments, 1m and 10m in the two sampling sites, Copacabana (Rey George Island) and Stinker Point (Elephant Island)

3. CONSIDERAÇÕES FINAIS

As análises de RNAseq realizadas nesse estudo demonstraram a expressão diferencial significativa de apenas um gene e somente em *Deschampsia Antarctica* Desv. O gene LOC_Os06g16380, recentemente descrito, se encontra inserido na mesma região que o gene *Hd1*, que em estudos com arroz apresentou respostas a nível de produtividade/rendimento e crescimento, assim como respostas ao fotoperíodo.

Através das análises de qRT-PCR foi possível verificar uma alta expressão do gene LOC_Os06g16380 nas amostragens mais próximas (1m) às áreas de reprodução de *Macronectes giganteus*, o que não ocorreu nas distâncias de 5 e 10 m em ambos os locais de estudo, Ilha Rei George e Ilha Elefante.

Nas análises de Nitrogênio mineral do solo verificaram-se altas concentrações de nitrogênio em forma de amônio (NH4) nas amostras mais próximas às colônias de reprodução de petréis (1m), sendo baixas as concentrações nas amostras de 10m e sem influência. Essa última análise confirmou e corroborou com nosso estudo de que quantidades de guano depósitos, a cada ano, na Antártica podem mudar as características químicas e orgânicas do solo, sugerindo que espécimes de *Deschapsia antarctica* para sobreviverem e suportarem essas grandes quantidades de nutrientes evoluíram seus mecanismos moleculares em resposta.

Os resultados não significativos com o musgo *Sanionia uncinata*, sugere que a ausência de raiz não permita a alta absorção de nutrientes, como acontece com as angiospermas (*D. antarctica*) que, evolutivamente, desenvolveram raízes que desempenham essa função. Além disso, esse resultado pode ter ocorrido devido a plasticidade fenotípica do musgo associada com sua ótima amplitude ecológica, nos levando a acreditar que o musgo não seja ideal para esse tipo de análise.

4. ANEXOS

NORMAS DE SUBMISSÃO NA REVISTA NATURE PLANTS

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