UNIVERSIDADE FEDERAL DO PAMPA

MÔNICA MUNARETO MINOZZO

ANÁLISE TRANSCRICIONAL DE *PHYSCOMITRIUM ACUTIFOLIUM* BROTH. POR MEIO DA TÉCNICA DE RNA-SEQ: UM ENFOQUE SOBRE O ESTRESSE POR FRIO EM PLANTAS.

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Dissertação apresentada ao programa de Pós-graduação *Stricto Sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Filipe de Carvalho Victoria

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Dedico este trabalho aos meus pais Almir e Gilsane pelo incentivo e amor incondicional

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RESUMO

Os estresses abióticos são responsáveis pela indução de adaptações em plantas. Estas quando submetidas ao estresse, respondem através de mecanismos de sinalização nas rotas fisiológicas, desencadeando um processo de aclimatação. Entretanto, nem sempre este potencial de adaptação é expresso, mas se persistir ao longo do desenvolvimento da planta, torna-se uma adaptação oriunda de mudança genética. Consequentemente, essas alterações incipientes possam ser identificadas em nível transcricional, os precursores de algumas alterações genéticas importantes tais como *splicing* alternativo. Em ambientes polares a expressão de genes permitiu a adaptação das plantas a temperaturas de congelamento. Entre estas plantas estão os musgos, presentes nos ambientes de climas contrastantes, isto sugere que estes organismos tenham plasticidade fenotípica e genotípica. Ainda que haja poucos estudos destes organismos em relação a diferentes agentes estressores, é amplamente difundido que estes possuem potenciais de resistência aos estresses ambientais. Para descobrir estes potenciais de resistências é necessário estudar os genes relacionados especificamente com o fator estressor em questão, neste caso o estresse ao frio. Para tanto é necessário um processo de sequenciamento dos genes expressos quando a planta é submetida ao estresse. Neste estudo foram realizados testes com explantes cultivados *in vitro* do musgo *Physcomitrium acutifolium* Broth. em diferentes temperaturas, com 6 tratamentos variando de 0 a 25 ºC, seguidos das análises fenotípicas e posteriormente genômicas, incluindo processo de sequenciamento e identificação de genes expressos. Os resultados sugerem relação do estresse por baixas temperaturas e o potencial de expressão de genes relacionados ao estresse por frio neste musgo, principalmente por uma identificação de maiores ocorrências de *splicing* alternativo nas plantas cultivadas a temperatura mais baixa testada. Assim, o uso potencial de espécies de musgo em estudos relacionados à resistência ao congelamento em plantas, torna-se como uma alternativa interessante em processos de biotecnologia vegetal.

Palavras-chave: Estresse abiótico, potencial anticongelante, sequenciamento genômico.

ABSTRACT

The abiotic stresses are responsible for inducing adaptations in plants. When subjected to stress, plants respond by signaling mechanisms in physiological pathways, starting a process of acclimatization. However, not every adaptation potential is identified in phenotypic level, but if the selection pressure led by the stress persist over plant development, there may be a change in genotypic level. Consequently, these incipient changes can be identified in transcriptional level, the precursors of some important genetic changes such as alternative splicing. In polar environments are noted that the plants were adapted to survive at low temperatures, this adjustment is related to the expression of genes, which confer them resistance to freezing. Among these plants are mosses, present in the environments of contrasting climates, this suggests that these organisms have phenotypic and genotypic plasticity. Even if there are few studies of these organisms in relation to different stressors, we know that these have the potential for resistance to environmental stresses. To discover these potential resistance is necessary to study the genes specifically related to the stressor in question, this study is the cold stress. Then you need a sequencing process is necessary genes expressed when the plant is subjected to stress. In this study were performed tests with cultured explants in vitro moss *Physcomitrium acutifolium* Broth. at different temperatures, with 6 treatments ranged from 0°C to 25°C, followed by phenotypic analysis and subsequently transcriptome analysis based in RNA sequencing process aiming to identify a differential gene expression. The results suggest stress relationship for low temperatures and the potential for expression of genes related to cold stress in this moss, mainly by an identification of a higher occurrences of alternative splicing in plants growing at lowest temperature tested. Thus, the potential use of moss species in studies related to plant resistance to freeze temperatures becomes as an interesting alternative in plant biotechnology processes.

Keywords: Abiotic stress, antifreeze potential, genomic sequencing

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1. INTRODUÇÃO GERAL

Os estresses abióticos induzem os processos responsáveis pelas adaptações nas plantas para garantir sobrevivência, estas podem gerar mutações no genoma. Este mecanismo de adaptação para tolerância ao estresse, acontece através das rotas fisiológicas e sinalizações celulares, que ocasionam proteção de membranas e proteínas, controle de transcrição, produção de proteínas, regulação de antioxidantes, radicais livres, acúmulo de solutos, proteínas envolvidas na proteção celular e nos fatores de transcrição. Estes dois últimos controlam os conjuntos específicos de genes regulados por estresse (BOHNERT; NELSON; JENSEN, 1995; GUPTA; DESWAL, 2014; SHAO et al., 2007; WANG; VINOCUR; ALTMAN, 2003; ZHANG, 2004; ZHU, 2001).

Assim ocorre a expressão gênica, nela existe um grande número de fatores de transcrição no genoma da planta, a maioria pertencentes a algumas grandes famílias de multigenes que respondem diferentemente a vários estímulos de estresse. Entretanto, alguns genes de resposta ao estresse podem partir do mesmo fator de transcrição, indicando sobreposição dos perfis de expressão gênica que são induzidos em resposta a diferentes estresses (MIURA; FURUMOTO, 2013; SEKI et al., 2003; SEO; PARK; PARK, 2013; THOMASHOW, 1999, 2010; TRAPNELL et al., 2012; VAN BUSKIRK; THOMASHOW, 2006).

As plantas que sobrevivem nas regiões polares tiveram que se adaptar para resistir as baixas temperaturas (BRAVO, 2005). A identificação de genes expressos em diferentes temperaturas que respondem ao estresse pelo frio nas plantas, podem ser reconhecidos pela técnica de sequenciamento genômico, juntamente com testes em diferentes temperaturas e comparação com outros organismos modelo (JAN; UL-HUSSAIN; ANDRABI, 2009).

Os musgos são organismos presentes tanto em regiões tropicais como polares, facilitando estes estudos de temperaturas contrastantes, entretanto, ainda temos poucos resultados à nível genômico com testes em musgos sobre estresses

abióticos causados por temperatura (BEIKE et al., 2015; CHANG; LIN; TU, 2014; LIU et al., 2013).

Contudo, são bastante difundidos estudos utilizando musgo como organismo modelo, em geral a espécie modelo utilizada é *Physcomitrella patens* (Hedw.) Bruch & Schimp, família Funariaceae, cujo genoma já sequenciado possibilita comparações com demais musgos (RESKI et al., 1994; RESKI; FRANK, 2005).

A espécie de musgo *Physcomitrium acutifolium* Broth., também da família Funariaceae, é nativo do Rio Grande do Sul, presente em regiões de Floresta Atlântica do Brasil, abrangendo os estados do Rio de Janeiro até o Rio Grande do Sul. Presente em ambientes de altitude de 0-200 m, que possuem clima úmido, com chuvas distribuídas quase uniformemente ao longo do ano, está na lista de espécies vulneráveis (VU). (COSTA et al., 2011; COSTA et al., 2005). Este possui rápido desenvolvimento para realização dos testes de cultivo *in vitro* em laboratório e possibilita a posterior comparação genômica com o organismo modelo *Physcomitrella patens*.

Considerando o fato de que os musgos apresentam desenvolvimento em temperaturas adversas e a possibilidade destes expressarem respostas fenotípicas ou genéticas com o estresse causado por estas flutuações de temperatura, realizou-se este estudo. Para o qual foram utilizados explantes do musgo *Physcomitrium acutifolium* cultivados *in vitro*, em diferentes temperaturas (0 a 25 ºC) para análise fenotípica e o sequenciamento de transcritos, buscando a identificação dos genes expressos sobre estresse abiótico por frio. A espécie escolhida ainda não foi estudada sob o ponto de vista da biologia molecular, sendo o presente estudo inédito para este modelo biológico.

1.1 OBJETIVO

Investigar os efeitos causados pelo estresse abiótico pela variação de temperatura de 0 a 25ºC, a partir da plasticidade fenotípica e expressão gênica na espécie de musgo temperado *Physcomitrium acutifolium* Broth.

1.2 HIPÓTESE

Plantas submetidas ao estresse abiótico por temperatura de congelamento respondem com expressões gênicas de proteínas de resistência ao frio e possivelmente de anticongelante.

2. ARTIGO: ENHANCED ALTERNATIVE SPLICING UNDER FREEZING TEMPERATURES AS SURVIVING STRATEGY IN *Physcomitrium acutifolium* BROTH.

(Submetido para revista Nature Plants)

Enhanced alternative splicing under freezing temperatures as surviving strategy in *Physcomitrium acutifolium* Broth.

Mônica M. Minozzo, Anthony D. M. Barboza, Luiz F. W. Roesch, Cristiane B. D'Oliveira, Antonio B. Pereira & Filipe C. Victoria

Alternative splicing shows that plants present potential tolerance to abiotic stress factors, though these potential is not always expressed. In plants from polar environments, studies have demonstrated that successful survivals express genes conferring resistance to freezing. To discover potential alternative splicing in a survival plant exposed to a particular type of abiotic stress, a sequencing strategy, based on analysis of expressed genes, is necessary. Such approach allows discovering those plant strategies to survive to a particular type of stress. Therefore, the present study aimed to identify the potential alternative splicing of a basal plant species for surviving under different temperature treatments (ranges from 0 to 25 ºC), using the moss *Physcomitrium acutifolium* as a model for genomic analyses. Phenotypic analysis were performed followed by a RNA-Seq in an ION PGM platform. The results suggest a relationship with low temperatures and the protein kinase-like gene expression, mainly by a identification of a higher occurrences of alternative splicing of genes related with these onthology, in plant growing al lowest temperature tested.

Over time plants undergone abiotic changes often leding to genotypic and phenotypic level adjustments to ensure survival. Temperature is one of these abiotic factors that cause changes. Many studies have been conducted on stress factors in plants to evaluate the potential for resistance to freezing and describe the molecular mechanism related (BRAVO, 2005; GUPTA; DESWAL, 2014; MIURA; FURUMOTO, 2013). The potential antifreeze resistance, also known as cold stress resistance can be classified, as chilling (0 - 15 °C) and freezing (<0 °C) stresses(THOMASHOW, 1999). Some plants from temperate regions exhibit a variable degree of chilling tolerance and can increase their freezing tolerance during exposure to chilling and non-freezing temperatures, for example *Arabidopsis thaliana* (L.) Heynh, known for exhibit a cold acclimation response(THOMASHOW, 1999). The plant growth and development are influenced by temperature changes and in response to this, different levels of gene regulation are modulated in the cell(CHANG; LIN; TU, 2014).

The genetic potential for responding to abiotic stresses in plants can be assessed by new techniques based on genomics(BEIKE et al., 2015; SEO; PARK; PARK, 2013; VICTORIA; DA MAIA; DE OLIVEIRA, 2011). Among those techniques, the genomic sequencing has been used to discover regions with potential response for cold stress however; to found these regions it's primarily necessary to know the transcription factor. The transcription factor is a critical component of the gene regulatory networks that mediate virtually all aspects of plant growth and developmental processes. The alternative splicing of primary transcripts evolved to overcome the limited coding capacities of eukaryotic genomes by producing multiple proteins from a single gene and enhance the transcriptome diversity and proteome plasticity(SEO; PARK; PARK, 2013). This alternative splicing is a widespread mechanism in eukaryotes that generates two or more mRNAs from the same precursor mRNA (pre-mRNA) by using different splice sites(CHANG; LIN; TU, 2014).

This work aims to increase the understanding of the mechanisms and plants potential for cold stress, based in the transcriptional analysis of a basal plant species, such as mosses. Mosses represent the oldest living clade of land plants, in this sense they are organisms with great potential for studies with plant biotechnology. *Physcomitrium*

acutifolium Broth. was chosen as a model because this moss presents fast growth in tropical and temperate environments, allowing to test its adaptation to the cold environment. Furthermore, this species belongs to the same family of the model species *Physcomitrella patens* (Hedw.) Bruch & Schimp (Funariaceae), it has been used for advance in plant biotechnology as model organism and its genome was already sequenced.

Results

Phenotype analyses. Compared to the extreme treatments (0, 5 and 25ºC) biomass increments were observed in treatments under 10, 15 and 20 ºC (Table 1). The gametophyte developed better at 15, 20 and 25ºC respectively. Under the cold temperatures (0 and 5ºC) the gametophyte did not developed and under 10 ºC, just 8 gametophytes were developed. The green color is related with a better photosynthesis status. The data on Table 1 showed that under extreme temperatures oxidation were observed on the explants, while treatments under 10, 15 and 20 º presented homogeneous green color indicating a better photosynthesis status in less extreme temperatures.

Differential expression analysis. To estimate the number of genes that were differentially expressed in the moss protonemata, we first normalized the gene expression values using a variation of the FPKM method (Fragments Per Kilo-base of mRNA length per Million mapped reads), using the Cufflink tool(TRAPNELL et al., 2012). The transcriptome data showed a large number of unique reads at 0 ºC and 20 ºC (Figure 1). Such expression level contrasted with the other treatments, suggesting that extreme temperatures applied in this experiment were suitable to find responsive genes to temperature stress factors.

The molecule Phpat.013G040900 presented high levels of expression within 0, 10 and 15 ºC (Figure 2). This molecule encodes to Phenylalanine and histidine ammonia-lyase (PAL), a protein related to abiotic and biotic stress in plants[10]. The ontology of the differentially expressed genes found in lower and higher temperatures were related with biological process components. The most expresses genes in our treatments presented semantic similarity with genes involved with phosphorylation proteins with kinase activity.

The genes differentially expressed under 0, 10 and 20 ºC, have mutual promoters, however at 20 ºC less promoters were observed when compared with lower temperatures. High numbers of splicings were also observed at the lower temperatures (Figure 4). The differential expression analysis suggested that moss species presented potential for supporting cold temperatures.

Discussion

The phenotypic analysis indicated that the temperatures variation applied in our experiment caused stress and in some freezing temperatures the genetic mechanisms for cold stress resistance were not enough to guarantee the explant survival. On the other hand, considering the use of a tropical moss as model, the stress caused at room temperature (25 ºC) was not expected. Such result indicated that temperatures around 10 at 20 ºC could be considered appropriated for better plant development.

The transcriptome analysis showed that the moss tested within this experiment presented genotypic potential for cold stress resistance however, such resistance did not present large plasticity since some explants died at freezing temperatures and did not developed well at room temperatures. Perhaps, this fact should have been developed due the needs for maintenance of living cells in extreme temperatures. This moss species is adapted to the temperate climate so the results only suggest the action of these genes in the cold adaptation process in plants(THOMASHOW, 1999).

The expression of the molecules Phenylalanine and histidine ammonia-lyase (PAL) was higher in cold temperatures (0, 10 and 15 ºC) suggesting potential resistance to cold[11], but lack of resistance to freezing temperatures. The results also showed a relationship with a molecule involved in phosphoryation of proteins, such as kinase activity, suggesting a connection between cold temperatures and photosynthesis. Such connection was already verified with concentration of sucrose in your freezing resistance in *Polytrichum juneperinum* Hedw and others plant species[12-14]. The phosphorylation of proteins in response to cold and the suppression of proteins with phosphatase activity may also provide a means for the plant to sense low temperature. Although most alternative splicing events have not been characterized in plants, several genes encoding protein kinases, transcription factors and splicing regulators have demonstrated the centrality of alternative splicing in the fine-tuning for abiotic stress responses[15]. These results suggests an existence of a mechanism for the differential response to cold resistance since the earliest days of colonization of plants to terrestrial environment, based in the phosphorylation of protein Kinase-like molecules.

The higher number of promoters and splicings found in plants growing in lower temperatures confirms other results that suggest the cold resistance potential in moss species, but up to date none of those studies provided information about antifreeze potential in mosses. Alternative splicing provides proteome diversity and, thus, expands the repertoire of gene/protein activities in response to developmental and environmental cues[16-17]. An interesting observation is that alternative splicing is often responsive to cold stress in plants, being cold-responsive gene regulation and alternative splicing frequently associated with each other in plants[18-19]. For mosses, these relationship were not established yet, since only splicing of heat-sensitive genes were studied in mosses(CHANG; LIN; TU, 2014). Therefore, the occurrences of kinase-like putative proteins were identified in *Pohlia nutans* (hedw) Lindb. transcriptome profiling from plants sampled at Antartic region, showing that molecules also plays an important role in the cold stress tolerance in mosses[6, 20].

Alternative splicing can be explored as means of elaborate control of transcription factor activities in crop plants based in biotechnological processes. The alternative splicing patterns through mutations in splice sites can be applied for modifying plant development and responses to environmental stresses. Knowing how this gene machine tool works on basal plant species may be useful for proposing genetic engineering strategies in a more simple way and can be applied to different groups of terrestrial plants, since they derived from the mosses lineage. Modulations of splicing factor activities would be an alternative approach to precisely control plant functions for improved stress tolerance.

Methods

The experiment was conducted in the Antarctic Plants Studies Core laboratory and Center for Interdisciplinary Research in Biotechnology (CIPBiotec) at Federal University of Pampa – Campus São Gabriel.

Plants materials and Growth Conditions. Fully developed *Physcomitrium acutifolium* plants were indentified and collected in São Gabriel municipality 30º 20' 5.11'' S, 54º 19' 6.90'' W) during the winter. Plants that possessed well developed sporophytes were selected the for the experimental procedures. Entire plant samples were deposited in the Bruno Edgar Irgang herbarium (HBEI), under the voucher number 47 (Bryophyte collection).

Fresh unopened sporophytes were surface sterilized by dipping in 25% commercial bleach (8% active NaOCl) for 3 minutes, and thoroughly rinsed in sterile distilled water[21]. The cap was then removed and the spores released on the nutrient medium. To obtain a minimal biomass necessary for further procedures, the spores were cultivated in the MS culture medium (Sigma-Aldrich), with pH adjusted for 5.8 and solidified in 7.0 g/L^{-1} of agar-agar (Vetec). Cultures were grown until showing the primary protonema in a photoperiod chamber at 25±1ºC under long-day conditions (16 h light/ 8 h dark) supplied by cool-white fluorescent tubes at a photon flow rate of 40-50 mol m^{-2}

s[1]. Three protonemata explants were transferred to Petri's dishes, with three repetitions for each one (Figure 5). The transference of explants were carried in a laminar flow hood to the maintenance of the axenic conditions of the experiment. The temperatures tested were: 0, 5, 10, 15, 20 and 25 ºC. The explants were cultivated during 30 days. After this period, the explants were evaluated based on the increment of biomass, number and development of gametophytes regenerated and oxidation. The biomass increment and the number of gamethophytes regenerated where evaluated in each treatment proposed, and a test of comparison of means was performed using the Tukey test (5% of probability) with the Statistix 9.0 for Windows software.

RNA Extraction. The protonemata obtained in each treatment were used for the total RNA extraction. One RNA extraction was performed for each treatment with three replicates (N = 18) using the Plant/Fungi RNA Purification Kit (Norgen Biotek, USA) according to the manufacturer instructions. The amount and quality of total RNA was measured by spectrophotometry using a NanoVue™ Plus Spectrophotometer (GE Healthcare).

mRNA Enrichment. Total RNA samples were subjected to enzymatic digestion of DNA using the TURBO DNA-free™ Kit (Ambion, USA) according to the manufacturer instructions. The depletion of cytoplasmic (5S, 5.8S, 18S, and 28S) and mitochondrial (12S and 16S) ribosomal RNA (rRNA) was performed with the RiboMinus ™ Eukaryote System v2 (Ambion, USA) following the manufacturer instructions and the mRNA recovered was was quantified by fluorometry using a Qubit – RNA Assay Kit (Invitrogen, USA).

Library preparation and Sequencing. Eighteen libraries were generated by using the Ion Total RNA-Seq Kit v2 kit (Ambion, USA). Ion OneTouch™ 2 System and Ion PGM™ Template OT2 400 Kit Template were used for library preparation and the sequencing was performed using Ion PGM™ Sequencing 400 on Ion PGM™ System using two Ion 318™ Chip v2 (nine samples loaded per chip). A total of 12.378,84 reads were generated for the 18 samples.

Assembly and mapping of transcripts. RNA-seq reads for each library were mapped independently using TopHat2 against the Physcomitrella patens genome build v3.0. Gene and isoform expression levels were calculated by running the Cufflinks tools (Cufflinks2, CuffMerge and Cuffdiff2) on the alignments from TopHat and the P. patens coding genes v3.1. All analysis was performed in Galaxy platform from Galaxy Rätsch Lab (galaxy.cbio.mskcc.org/). The P. patens genome and gene annotation were downloaded from Phytozome V10.1 (phytozome.jgi.doe.gov).

The statistics and graphical analysis of the results were held with R Program (version 3.1.1) with CummeRbund extension[22].

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Contributions
M.M.M carried out experiments in *Physcomitrium acutifolium*, 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. L.F.W.R. designed and carried out the RNA-Seq sequencing. C.B.D. carried out the *P. acutifolium* collections. A.B.P. co-directed the project. F.C.V. conceived and co-directed the Project, designed and carried out the bioinformatic analysis and transcriptome assembly.

Competing interests
The authors declare no competing financial interests.

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Table 1. Average biomass obtained in moss cultivation after 30 days.

Treatment	0 ºC	5 ºC	10 ºC	15 ºC	20 ºC	25 ºC
Biomass (mg)	9.0 ^{B}	12.3 ^{B}	71.1 ^{A}	62.1 ^{A}	63.2 ^{A}	27.2 ^{B}
Gametophyte regenerated	0 ^{C}	0 ^{C}	8 ^{C}	133 ^{B}	336 ^{A}	50 ^{BC}
Oxidation	Yes	No	No	No	No	Yes

* Averages followed by the same letter in the same row do not differ by Tukey test (α=0,05).

Figura 1. Gene expression (total) in different treatments.

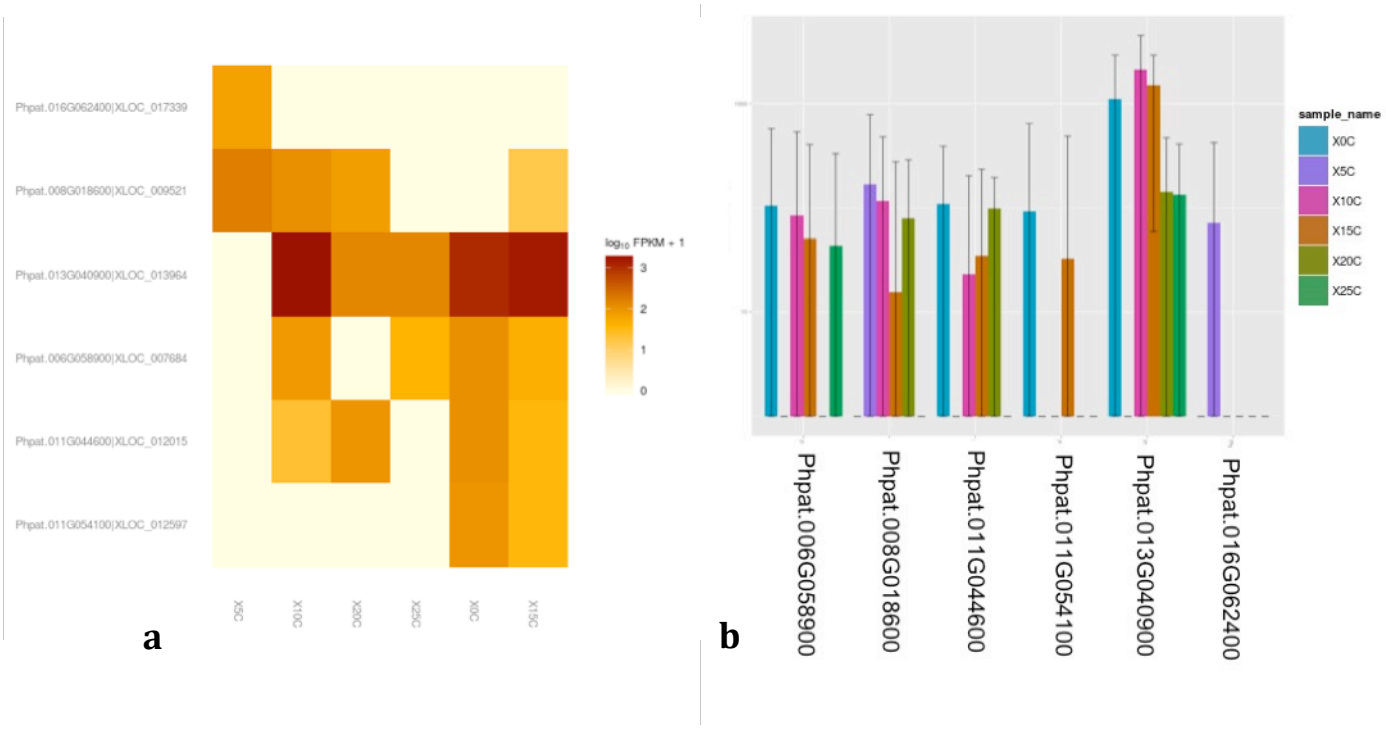

Figure 2. Genes expressions a,b. (**a**) Gene expression of 6 genes significant (0.05) in different treatments. (**b**) Heatmap of 6 genes differentially expressed (0.05) in different treatments.

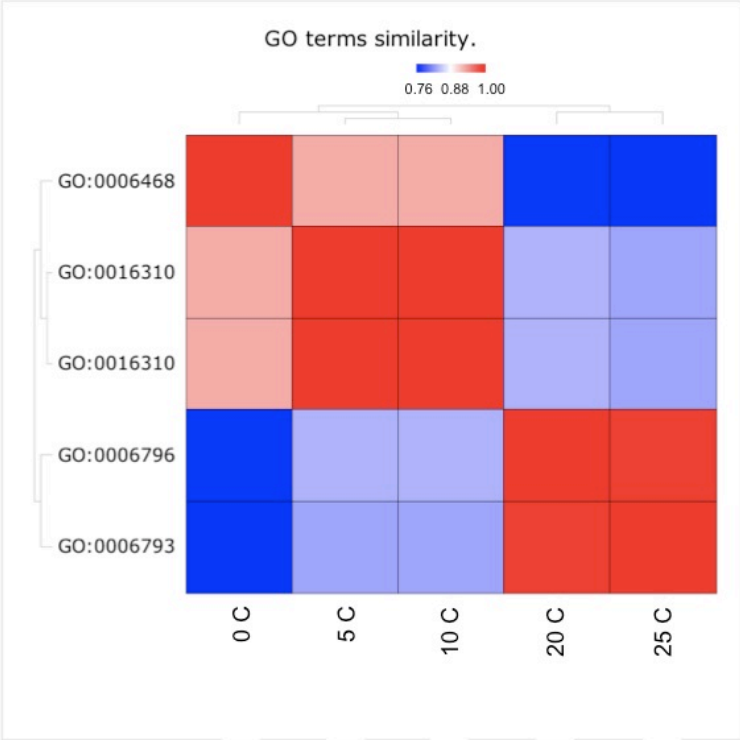

Figure 3. Biological process components in Gene Ontology.

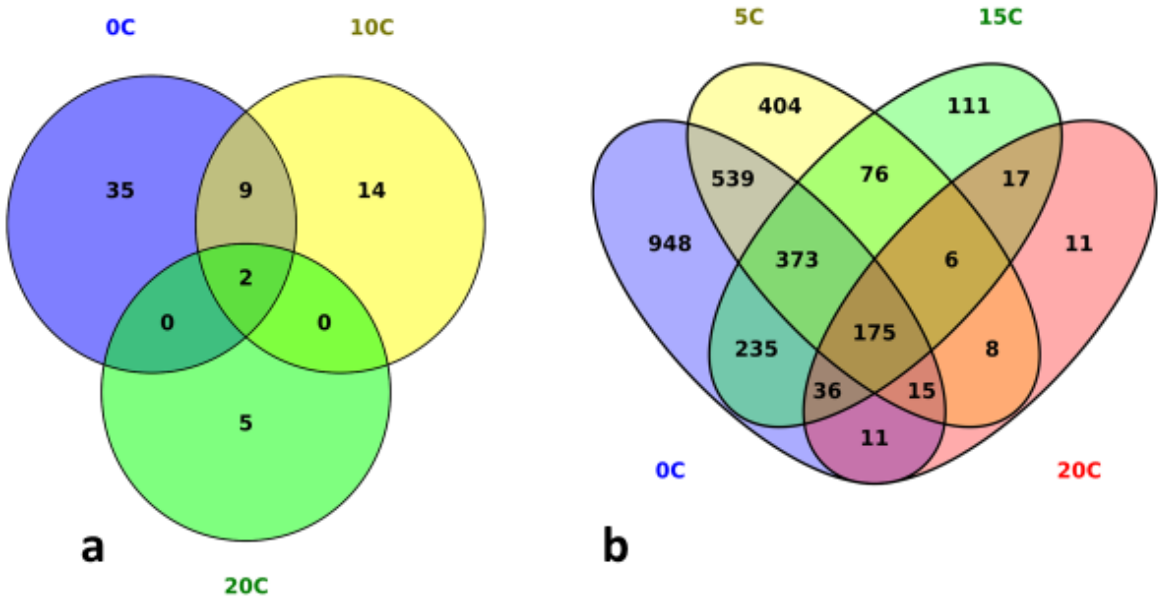

Figure 4. Venn Diagram a,b. The promoters found in treatment of 0, 10 and 20 ºC (**a**) and splicing significant number for each treatment (**b**).

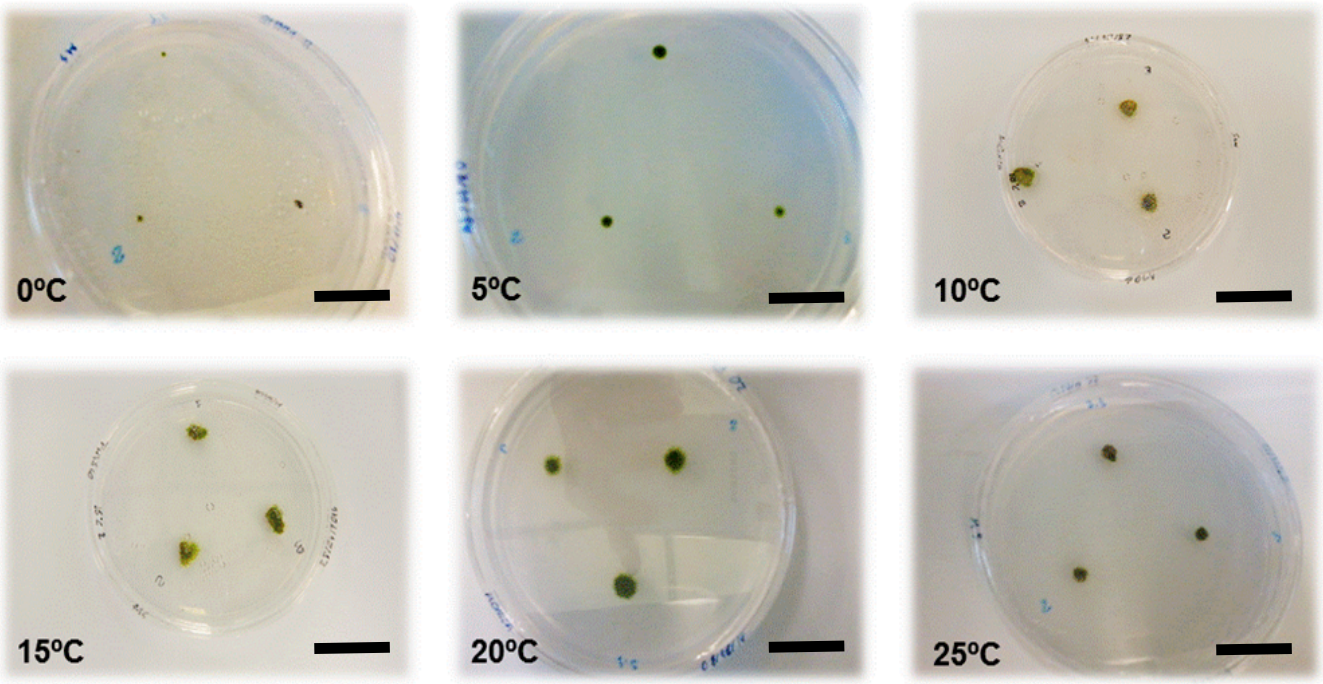

Figure 5. The explants at the completion of the experiment, in the all treatments after 30 days.

(Bars: 0 ºC = 18,70mm, 5 ºC = 17mm, 10 ºC = 27mm, 15 ºC = 27,87mm, 20 ºC = 20,23mm, 25 ºC = 22,09mm.)

3. CONSIDERAÇÕES FINAIS

O presente estudo corrobora a hipótese que *Physcomitrium acutifolium*, submetido a temperaturas contrastantes, reage com alternativas de expressões gênicas de acordo com as mudanças de temperatura.

Apesar da comprovação de potenciais gênicos para adaptações de resistência ao estresse por temperaturas próximas ao congelamento, não obteve-se certeza do potencial de resistência ao congelamento, o que necessita estudos mais direcionados que só serão possíveis a partir de maior conjunto de dados e posteriores testes em laboratórios.

Da mesma maneira que este potencial de adaptações que conferem resistência nas baixas temperaturas testadas, não há garantia que estas plantas aclimatadas irão sobreviver por longos períodos a estas temperaturas, pois ainda não é comprovado que esta esteja totalmente adaptada a esta nova condição antes estressora.

Como perspectivas futuras deste projeto, pretendemos realizar este mesmo estudo com uma espécie de musgo da Antártica, por ser espécie glacial e já proveniente do frio. Mas que também esteja presente em outros climas e possua proximidade genética com a espécie modelo, assim colaborando com a comparação da ação dos genes neste processo de adaptação. E a partir dos resultados obtidos, começar os experimentos de manipulação e transformação gênica para entender o processo fisiológico de resposta a este estresse abiótico.

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5. ANEXOS

5.1 NORMAS DE SUBMISSÃO NA REVISTA NATURE PLANTS

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- Figures divided into parts should be labelled with a lower case, bold **'a'** , **'b'** , et cetera, in the top left hand corner. Labelling of axes, keys and so on should be in 'title case' with no full stop. Units must have a space between the number and the unit, and follow the nomenclature common to your field
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- Figures should be prepared at the size you would expect them to appear in *Nature Plants*.

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5.2 CARTA DE SUBMISSÃO NA REVISTA NATURE PLANTS

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Manuscript #	NPLANTS-15050739
Current Revision #	0
Submission Date	18th May 15
Current Stage	Manuscript under submission
Title	Differential expression analysis reveals an enhancement of alternative splicing related with the freezing temperature in the moss Physcomitrium acutifolium growing in axenic conditions
Manuscript Type	Presubmission Inquiry
Corresponding Author	Dr. Filipe Victoria (filipevictoria@unipampa.edu.br) (National Institute of Antarctic Science and Technology for Environmental Research, Federal University of Pampa)
Contributing Authors	Miss Mônica Minozzo , Mr. Anthony Barboza , Ms. Cristiane D'Oliveira , Dr. Luiz Fernando Roesch , Dr. Antonio Pereira
Authorship	Yes
Abstract	The alternative splicing shows that plants have potential for tolerance of abiotic stress factors, though not always express these potentials. In plants from polar environments, studies have demonstrated that successful survivals of these are related with the expression of genes, which confer resistance to freezing. To discover potential alternative spliced into a plant survival of a particular type of abiotic stress, a sequencing strategy based on analysis of expressed genes is necessary to find those responsive to the kind of stress studied. Therefore, in the present approach, plants explants was been cultivate in the optimal condition to their development before begin temperature experiments and the sequencing process. Was aimed to identify the potential of alternative splicing of a basal plant species in the survival under different temperature treatments (ranges from 0 to 25 ºC), using the moss Physcomitrium acutifolium Broth. as a model for genomic analyses.
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Applicable Funding Source	No Applicable Funding

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