EVALUATION ON THE EXPRESSION OF HYDROLYTIC ENZYMES IN *TRICHODERMA HARZIANUM* UNDER VARYING LIGHT CONDITIONS

Rafaela R. Rosolen[1,2], Maria Augusta C. Horta[1], Danilo A. Sforca[1] & Anete P. de Souza[1,3*]

[1] *Center for Molecular Biology and Genetic Engineering (CBMEG), University of Campinas (UNICAMP), Campinas, SP, Brazil.*
[2] *Graduate Program in Genetics and Molecular Biology, Institute of Biology, UNICAMP, Campinas, SP, Brazil.*
[3] *Department of Plant Biology, Institute of Biology, UNICAMP, Campinas, SP, Brazil.*
[*] *Corresponding author's email address: anete@unicamp.br*

ABSTRACT

Trichoderma harzianum plays an essential role by efficiently producing and secreting carbohydrate-active enzymes (CAZymes). The expression of the genes that encode such hydrolytic enzymes is tightly controlled at the transcriptional level and dependent on the carbon source available in the extracellular environment. Additionally, studies have shown that abiotic factors, such as light, play a considerable role in the gene regulation of CAZymes. Therefore, the elucidation of the genetic mechanisms associated with light signals in the expression of hydrolytic enzymes is necessary and can help in bioprospecting enzymes from T. harzianum IOC-3844 (Th3844), a potentially hydrolytic strain. Herein, we cultivated Th3844 under different light conditions aiming to investigate the evaluation of the expression level of the target genes through reverse transcription-quantitative PCR (RT-qPCR). These genes included CAZymes, transcription factors, photoreceptors, and genes related to signal transduction pathways as well as the reference genes. Through the investigation of the genome structure by syntenic analysis and the expression level of target genes, this work aims to study the influence of light on the expression of hydrolytic enzymes in Th3844; therefore, generating data that can contribute relevant information to be considered in the selection and improvement of enzymes of Th3844.

Keywords: Light; CAZyme; *Trichoderma harzianum*; RT-qPCR; Reference genes.

1 INTRODUCTION

Saprophytic fungi contribute to carbon recycling by degrading lignocellulosic into fermentable sugars and ethanol. This is possible because their genomes encode a vast array of enzymes, including carbohydrate-active enzymes (CAZymes), which are produced and secreted according to the substrate on which they grow. In addition to nutritional components, in recent years light has emerged as a crucial environmental variable in modulating gene expression of hydrolytic enzymes in *Trichoderma reesei*, an important producer of industrial enzymes[1]. Eight proteins are considered to be responsible for light perception including the transcription factors (TFs) BLR1 and BLR2, the photoreceptor ENV1, two photolyases, a cryptochrome, a phytochrome and an opsin, which is however only present in the genome of *Trichoderma atroviride*, but not in *T. reesei*[1]. Heterotrimeric G-protein signaling and the cAMP pathway are also involved in the light-dependent regulation of enzymes of plant cell wall degrading[1]. Additionally, several genes previously known to regulate secondary metabolism in fungi, such as *vel1*, *lae1* or *ypr2* were identified as regulators of cellulase gene expression[1], hence supporting a connection between plant biomass degradation and secondary metabolism.

More recently, *Trichoderma harzianum* strains were explored for their enzymatic potential and were demonstrated to be useful for improving lignocellulosic conversion into sugars during second-generation ethanol (2G ethanol) production[2-4]. Previous studies have suggested the great potential of a strain from *T. harzianum* isolated from the Brazilian Amazon rainforest, namely IOC-3844 (Th3844), as hydrolytic enzyme producer when compared to another *Trichoderma* spp[3, 5]. Considering the great potential of Th3844 and the influence of light on gene regulation related to hydrolytic enzymes demonstrated for *T. reesei*, a better understanding of this process in such strain is important to expand its potential for bioprospecting of lignocellulolytic enzymes. In this study, we aimed to investigate the influence of light signals on the regulation of genes associated with the degradation of plant biomass Th3844. For this, such strain was cultivated under constant light or constant darkness and using cellulose or glucose as a carbon source. This two-dimensional analysis enables us to evaluate distinct regulation patterns in light and darkness and to discern induction-specific regulation that is independent of light. From the results, we hope to determine the influence of light on the production of hydrolytic enzymes in Th3844 and to contribute to its improvement by considering light effects.

2 MATERIAL & METHODS

The species originated from the Brazilian Collection of Environment and Industry Microorganisms (CBMAI), which is located in the Pluridisciplinary Center for Chemical, Biological, and Agricultural Research (CPQBA) at the University of Campinas (UNICAMP), Brazil. The identity of Th3844 was authenticated by CBMAI based on phylogenetic studies of their internal transcribed spacer (ITS) region, translational elongation factor 1 (*tef1*), and RNA polymerase II (*rpb2*) marker gene[6]. Th3844 was grown on potato dextrose agar (PDA) for 10 days at 28 °C to produce a sufficient number of spores as an inoculum for fermentation. Fermentation was performed in biological triplicates and was initiated with the inoculation of 10^7 spores /mL in an initial volume of 200 mL of pre-inoculum composed of 10 g /L crystalline cellulose or glucose, 1 g /L peptone, and 100 mL /L Mandels Andreotti (MA) minimal medium[7]. After 72 h of incubation, 50 mL of the pre-inoculum was used to inoculate 450 mL of fermentation solution in a 2 L Erlenmeyer flask. The composition of the fermentation volume varied according to the carbon source, which was either crystalline cellulose or glucose (10 g /L carbon source, 1 g /L peptone, 100 mL /L MA, 1 mL/L Tween, pH 5.3, with potassium biphthalate). After 96 h of growth in constant light (LED SLIM SMD 30W BIV 6500K IP66 BRONZEARTE) or in constant darkness

at 28 °C in a rotary shaker at 200 rpm, mycelia and supernatants were harvested. To avoid interference of light pulses with gene regulation, harvesting was done under a red safety light (darkroom lamp, Philips PF712E, red, E27, 15 W). The aqueous extract was frozen at −20 °C and the mycelium was quick-frozen in liquid nitrogen and stored at −80 °C.

Total RNA from 1 mg of each sample was extracted using TRIzol Reagent (Invitrogen, Karlsruhe, Germany). RNA sample integrity was determined by 1% (w/v) agarose gel electrophoresis, and purity and concentration were determined by a NanoVue spectrophotometer (GE Healthcare, Chicago, IL, USA). A Quantitec Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to perform reverse transcription of the extracted RNA samples. The cDNA mixture was diluted 1:20 and stored at -70 °C for subsequent reverse transcription-quantitative PCR (RT-qPCR) analysis. For the RT-qPCR analysis, we chose 30 genes, including CAZymes, TFs, transporters, photoreceptors, and proteins involved with the heterotrimeric G-protein and cyclic adenosine monophosphate (cAMP) pathways. Reference genes that were commonly used in eukaryotes were selected to screen for stable gene expression in Th3844 grown under the described light conditions. These candidate reference genes included *sar1*, *vma1*, *tbp*, *gapdh*, *tef1*, *rho*, and *rpl6e*, selected according to previously published reference gene analysis[8].

Primers were designed using the Primer3Plus[9]; the target amplicon sizes ranged from 120 to 200 bp, with an optimal annealing temperature of 60 °C and an optimal primer length of 20 bp. Amplification efficiency of each primer pair was evaluated by the standard curve method using serial dilutions of cDNA (1:10, 1:100, 1:1000, and 1:10000) as a template. Primers were considered efficient when they presented 90% ≥ and ≥ 110% and 0.95 ≥ r2 ≥ 1. Quantification of gene expression was performed by continuously monitoring SYBR Green fluorescence. The reactions were performed in triplicate in a total volume of 8 µL. Each reaction contained 4 µL of SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.3 µL of forward and reverse primers, 2 µL of diluted cDNA, and 1.4 µL of sterile water. Non-template controls (NTC) in which the cDNA was replaced with nuclease free water were also included for each primer pair to exclude possible contaminations The reactions were assembled in 384-well plates. RT-qPCR was conducted with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression will be calculated via the delta-delta cycle threshold method[10].

SimpleSynteny software (server version 1.6 – default parameters)[11] was used to compare clusters of genes physical proximity to the *blr1*, *blr2*, *clr1*, and *clr2* among different species of *Trichoderma* spp.

3 RESULTS & DISCUSSION

To investigate carbon source-specific gene regulation in Th3844, we will conduct RT-qPCR analysis of cultures grown on cellulose, an inducer of cellulase expression, and on glucose, a repressor of cellulase expression. Given the significant impact of light on gene regulation in *T. reesei*, including the regulation of CAZymes, we will also subject our cultures to constant light and constant darkness during cultivation. This experimental setup will allow us to assess carbon source-specific effects and determine whether these effects are influenced by light conditions. To achieve this, we will examine genes previously reported to be differentially regulated between light and darkness on cellulose and glucose in *T. reesei*[1]. Consequently, all samples obtained from light-exposed cultures will be treated as replicates in comparison to samples obtained from cultures grown in darkness.

After cultivating Th3844, we conducted RNA extraction from samples in biological triplicates for each growth condition, resulting in a total of 12 samples: 3 for cellulose under light, 3 for cellulose under dark, 3 for glucose under light, and 3 for glucose under dark. All RNA samples were standardized to 500 ng/µL for cDNA synthesis. To initiate the RT-PCR analysis, we initially assessed the primer efficiency for 35 genes, including the reference genes. Subsequently, eight primer pairs, including *ace3* (TF), *bgl1* (CAZyme), *each42* (CAZyme), *egl6* (CAZyme), *phr1* (photolyase), *phr1_cry* (photolyase), *pkr1* (protein kinase A), and *xyn2* (CAZyme), were excluded from the analysis due to inefficiency based on amplification efficiencies, correlation coefficients (R^2), and slope values.

Normalization of RT-qPCR data is typically accomplished using reference genes, which exhibit stable expression across various experimental conditions and serve to normalize the expression levels of target genes[12]. Despite its critical role and potential applications, reference genes for RT-qPCR analysis in Th3844 have yet to be evaluated under different light conditions, to the best of our knowledge. Therefore, following primer efficiency screening, we commenced the analysis by validating our chosen reference genes, sourced from existing literature. Our initial findings indicated that the expression levels of the genes *gapdh* and *tef1*, for example, varied across conditions, disqualifying them as suitable reference genes for our experiments. Consequently, we are currently exploring alternative reference genes. Once the reference genes are established, we will proceed to examine the expression patterns of the selected target genes.

Establishing syntenic relationships among groups of orthologous genes is crucial for deciphering molecular-level similarities and differences in genome evolution and species diversity through comparative genomics approaches[13]. When comparing syntenic genes among *T. harzianum*, *T. atroviride*, and *T. reesei*, notable differences were observed (**Figure 1**). Th3844 exhibited a genomic organization profile identical to Th0179, except for the absence of a gene encoding a protein related to ubiquitination, which was not present in Th0179. In Ta0020, four genes (a domain, a transporter, a putative methyltransferase, and genes related to ubiquitination and epigenetic control) from the cluster were not detected. Additionally, an inversion of genes encoding a putative methyltransferase and transmembrane domain relative to Tr0711 genome was observed. The genes conserved across all species analyzed suggest a potential association between their regulation and expression (**Figure 1**). Overall, synteny analysis revealed greater differences in relation to the *T. reesei* genome compared to the other strains studied. These findings could be attributed to gene loss and genomic modifications occurring in *T. reesei* lineages[14], potentially enhancing the production of enzymes involved in plant biomass degradation. Furthermore, these results align with the phylogeny of the genus *Trichoderma*, wherein *T. atroviride* is phylogenetically distant from *T. reesei*[14]. The results obtained from the syntenic analysis will be correlated with the gene expression data.

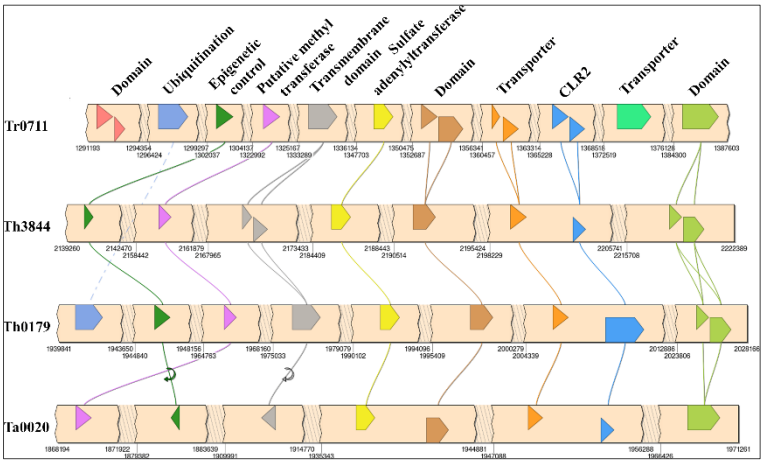

Figure 1 Comparison between the CLR2 gene cluster of Th3844 and those of other species of the genus *Trichoderma* spp. Arrows with the same color and pattern represent homologs. Tr0711: *T. reesei* CBMAI-0711; Th3844: *T. harzianum* IOC-3844; Ta0020: *T. atroviride* CBMAI-0020; Th0179: *T. harzianum* CBMAI-0179.

4 CONCLUSION

We aim to conclude the PCR analyses of the target genes and present findings on the influence of light on the expression of enzymes involved in plant cell wall degradation in Th3844 by the date of the congress.

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