Microbiological Research 227 (2019) 126296

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Hsp genes are differentially expressed during *Trichoderma asperellum* self-recognition, mycoparasitism and thermal stress

Thuana Marcolino Mota[a], Letícia Harumi Oshiquiri[a], Érica Camelo Viana Lopes[b], Jomal Rodrigues Barbosa Filho[a], Cirano José Ulhoa[a], Raphaela Castro Georg[a,*]

[a] Laboratory of Biochemistry of Microorganisms - ICB2, Room 211, Universidade Federal de Goiás, Campus Samambaia, 74001-970, Goiânia, GO, Brazil
[b] Applied Science Program for Health Products, Universidade Estadual de Goiás, Anápolis, GO, Brazil

ARTICLE INFO

Keywords:
Trichoderma
Mycoparasitism
Hsps
Heat stress

ABSTRACT

Heat shock proteins (Hsp) are important factors in the response of organisms to oscillations in environmental conditions. Although Hsp have been studied for a long time, little is known about this protein class in *Trichoderma* species. Here we studied the expression of *Hsp* genes during *T. asperellum* growth, and mycoparasitism against two phytopathogens: *Sclerotinia sclerotiorum* and *Fusarium oxysporum*, as well as during thermal stress. The expression levels of these genes were observed by real-time PCR and they showed to be differentially expressed under these conditions. We verified that the *TaHsp26c*, *TaHsp70b* and *TaHsp70c* genes were differentially expressed over time, indicating that these genes can be developmentally regulated in *T. asperellum*. Except for *TaHsp26a*, all other genes analyzed were induced in the post-contact condition when *T. asperellum* was cultured in a confrontation plate assay against itself. Additionally, *TaHsp26b*, *TaHsp26c*, *TaHsp90*, *TaHsp104a* and *TaHsp104b* were induced during initial contact between *T. asperellum* hyphae, suggesting that these proteins must play a role in the organism´s self-recognition mechanism. When we examined gene expression during mycoparasitism, we observed that some genes were induced both by *S. sclerotiorum and F. oxysporum*, while others were not induced during interaction with either of the phytopathogens. Furthermore, we observed some genes induced only during confrontation against *S. sclerotiorum*, indicating that the expression of *Hsp* genes during mycoparasitism seems to be modulated by the phytopathogen. To assess whether such genes are expressed during temperature oscillations, we analyzed their transcription levels during thermal and cold shock. We observed that except for the *TaHsp70c* gene, all others presented high transcript levels when *T. asperellum* was submitted to high temperature (38 °C), indicating their importance in the response to heat stress. The *TaHsp70c* gene was significantly induced only in cold shock at 4 °C. Our results show the importance of Hsp proteins during self-recognition, mycoparasitism and thermal stress in *T. asperellum*.

1. Introduction

The genus *Trichoderma* is the most studied group of biocontrol agents of plant pathogenic fungi. Its rapid mycelial growth, associated with high conidia production, synthesis of several antibiotics, and ability to live in different lifestyles (saprotroph, symbiont or mycoparasite) are characteristics that make it attractive for this purpose.

The most direct mode of action of *Trichoderma* action against phytopathogenic fungi is through the interaction known as mycoparasitism. Of all plant disease control mechanisms, this is the most complex and shows the greatest number of steps involved (Lima et al., 1998). *Trichoderma* act as antagonists against several plant pathogenic fungi, such as *Fusarium*, *Rhizoctonia*, *Phytium* and *Sclerotinia* species, pathogens that are of fundamental importance for agriculture (Thrane et al., 1997). The ability of *Trichoderma* to inhabit different environments is related to its capacity to overcome varied environmental conditions (Hermosa et al., 2004; Montero-Barrientos et al., 2008).

To adapt to oscillations in environmental conditions, the vast majority of organisms, including microorganisms, respond to these stimuli by altering cellular metabolism, and activating their defense mechanisms. Among the known stress defense proteins, Heat shock proteins (Hsps) have a prominent role, being fundamental in the response to varied environmental conditions (Georg and Gomes, 2007a; Seidl et al., 2009; Schmoll et al., 2016). Heat shock proteins are proteins responsible for the folding or re-folding of other proteins, and are extremely important under stress conditions (Lindquist, 1986; Lindquist

* Corresponding author.
E-mail address: rcgeorg@ufg.br (R.C. Georg).

https://doi.org/10.1016/j.micres.2019.126296
Received 12 November 2018; Received in revised form 4 June 2019; Accepted 1 July 2019
Available online 02 July 2019
0944-5013/

and Craig, 1988; Vierling, 1991; Plesofsky-Vig and Brambl, 1993). These proteins are grouped into families: sHsp (Hsp of small molecular weight), Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100, according to their amino acid sequences and molecular weights (in kD) (Jäättelä and Wissing, 1992; Fuller et al., 1994).

Hsps play different roles in fungal biology; the expression of these proteins is variable and occurs both in response to stress and during basal metabolism. Studies have demonstrated the crucial role of these proteins in fungi, whether in morphogenic change, adaptation to stress, or antifungal resistance (Tereshina, 2005; Lamoth et al., 2015). Indeed, there are reports in the literature suggesting the involvement of Hsps in mycoparasitism by *Trichoderma* (Seidl et al., 2009; Steindorff et al., 2012). However, the mechanism by which these genes participate in phytopathogen interaction have not been studied in detail until now. In addition, studies indicating the involvement of *Trichoderma* Hsps in mycoparasitism are based on large-scale techniques, and these data have not been confirmed by northern blot or RT-qPCR.

The few functional reports that exist in the literature about Hsps from *Trichoderma* are mainly summarized in evaluation of the over-expression of *Trichoderma* Hsps in plants or in *Trichoderma* itself and acquisition of increased tolerance to stresses (Montero-Barrientos et al., 2007, 2008; Montero-Barrientos et al., 2010). Even so, little information exists regarding the study of *Trichoderma Hsp* genes under environmental stresses as well as during growth under non-stressful conditions. To the best of our knowledge, functional studies on the importance of Hsps during the confrontation with phytopathogens or during *Trichoderma* self-recognition are nonexistent.

Considering the importance of this class of proteins for cellular homeostasis we evaluated the expression levels of nine genes encoding Hsps to better understand the role of *Hsps* genes during *T. asperellum* growth, mycoparasitism, and in response to temperature oscillations. We observed that *Hsps* genes are differentially expressed according to *T. asperellum* growth conditions, being expression levels not correlated to Hsp family. We also verified that the *T. asperellum* self-recognition process modulates the expression of all *Hsps* genes analyzed. In this way, this work opens new perspectives in the study of *Trichoderma* self-recognition mechanism.

2. Materials and methods

2.1. Maintenance of isolates

The isolates of *Trichoderma asperellum* (TR356), *Sclerotinia sclerotiorum* (SS26) and *Fusarium oxysporum* (FO344) belonging to the collection of the Enzymology Laboratory of the Federal University of Goiás (UFG) were used. Fungi were maintained with periodic culture in MYG medium [5% (w/v) malt extract, 0.25% (w/v) yeast extract, 1% (w/v) glucose and 2% (w/v) agar].

2.2. Sequence analyses

The amino acid sequences of the *T. asperellum* Hsp proteins (http://genome.jgi.doe.gov/Trias1/Trias1.home.html) (Druzhinina et al., 2018) were aligned against all proteins predicted in the genomes of: *T. atroviride* (https://genome.jgi.doe.gov/Triat1/Triat1.home.html), *T. harzianum* (https://genome.jgi.doe.gov/Triha1/Triha1.home.html) (Druzhinina et al., 2018), *T. reesei* (https://genome.jgi.doe.gov/TrireRUTC30_1/TrireRUTC30_1.home.html) (Jourdier et al., 2017; Koike et al., 2013) and *T. virens* https: //genome.jgi.doe. gov / TriviGv29_8_2 / TriviGv29_8_2.home.html), using the Blastp tool (Altschul et al., 1997). Hsp amino acid sequences identified after the comparison were posteriorly aligned using Clustal Omega (Sievers et al., 2011), and their promoter sequences were retrieved from the genome database to predict regulatory elements. Using MultiLoc2 (https://abi.inf.uni-tuebingen.de/Services/MultiLoc2) (Blum et al., 2009) it was possible to predict the subcellular localizations of all 9 proteins examined.

Table 1
Sequences of oligonucleotides used for analysis of gene expression in real-time PCR.

Name	Sequence (5'→3')	Bases	Tm °C
Actin R	ACG TAG GAG TCC TTC TGA CCC ATA	24	58.6
Actin F	CGA CAA TGG TTC CGG TAT GTG CAA	24	59.8
TriasHsp26aF	AGT TGA GCG CAC TTA CAC AG	20	55.3
TriasHsp26aR	ATT GCG CCA CTC ATT GAA GC	20	56.6
TriasHsp26bF	AAG AAC CTG TGC GCA GAA AC	20	56.1
TriasHsp26bR	ACG ACT TCA AAG CCC GAT TC	20	55.4
TriasHsp26cF	AAG TCG CAA AGT CCT CTG AGG	21	56.9
TriasHsp26cR	TGA AAT TGG CCG ACA CGT TG	20	56.4
Triashsp70 aF	TTC AGT CGC CAA GTT TGC TC	20	55.9
Triashps70 aR	TCT GCG TTC TCA ATG ATG CG	20	55.7
Triashsp70 bF	TCA ACG AGT TCA AGC GCA AG	20	56.0
Triashsp70 bR	AGA AGA AAG GGT TCG CTT GG	20	55.2
Triashsp70 cF	TTG ATT GGT CGC AAG TTC GC	20	56.4
Triashsp70 cR	ACC TGA ACA ATG GGC TTT CC	20	55.6
TriasHsp90F	ACC TGC GCA TTG ACA TCA TC	20	55.9
TriasHsp90R	AGG TTG TTG ACA AGG TCA GC	20	55.4
TriasHsp104aF	TGC AAG TCA CCT GAA ACT GC	20	55.9
TriasHsp104aR	TTT TTC TCG GCG CTC TTT GC	20	56.7
TriasHsp104bF	ATG CCA TCA ACG CAG GAA AC	20	56.4
TriasHsp104bR	GCC ACA TCT GCA GCA TTC TTC	21	56.9

2.3. Primer design

The primers for real-time (RT-qPCR) reactions were designed with the Primer3 program (Untergasser et al., 2012; Koressaar and Remm, 2007). The nucleotide sequences of the primers used in this work are presented in Table 1.

2.4. Dual culture test

The interactions between *T. asperellum, Sclerotinia sclerotiorum* and *Fusarium oxysporum* phytopathogens were evaluated using the paired culture test. All isolates were cultured on MYG medium in Petri dishes.

Colonized 5 mm diameter discs from *T. asperellum* and from phytopathogens were transferred to 9 cm plates containing MYG medium previously covered with sterile cellophane membranes. The discs were arranged on opposite sides of the plate, at 1 cm from the edge. The plates were incubated in a BOD incubator at a temperature of 28 °C, and photoperiod of 12 h (Steindorff et al., 2014). For RNA extraction, the mycelia of *T. asperellum* from the front edge of these colonies, cultivated alone, with itself, and with phytopathogens *F. oxysporum* and *S. sclerotiorum,* was scraped from the surface of the plate using previously autoclaved spatulas. The scraping of the mycelia was carried out during the pre-contact phases (about 1.5 cm separation from hyphae), contact (visually determined contact with hyphae) and post-contact (after five days of growth). Scraping of *T. asperellum* mycelia was performed with a safety margin to avoid contamination with mycelia from the challenging fungi (Supplementary data 1). The *T. asperellum* mycelia scraped under the conditions cited above were wrapped in sterile aluminum foil and frozen at −80 °C. All experiments were performed with three biological replicates.

2.5. Cultivation in liquid medium

For the thermal stress test, cultures were performed on 9 cm plates containing MYG medium. After growth for seven days, *T. asperellum* mycelium was scraped from plates (¼ of mycelium area) and inoculated into 250 ml flasks containing 50 ml TLE [1.0 g L^{-1} Bactopeptone, 0.3 g L^{-1} Urea, 2.0 g L^{-1} KH_2PO_4, 1.4 g $L^{-1}(NH_4)2SO_4$, 0.3 g L^{-1} $MgSO_4$ $0.7H_2O$, 0.2 g L^{-1} $CaCl_2$ $0.2H_2O$, 2% (v/v) trace element solution, pH 5.0] with 2% (w/v) glucose. The flasks were incubated on a rotating shaker at 28 °C and speed of 180 rpm.

2.6. Thermal stress

After 48 h growing in TLE medium on a rotary shaker, vials containing mycelia were conditioned at 38 °C for 1, 2 or 4 h. Another thermal stress test was performed where, after 48 h of growth in TLE medium, vials containing mycelia were conditioned at 4 °C, 10 °C and 32 °C for 1 h. The mycelia from each treatment and controls (28 °C) were vacuum filtered, washed in 0.9% saline solution and frozen at −80 °C for RNA extraction. All experiments were performed with three biological replicates.

2.7. RNA extraction and cDNA synthesis

Total RNA was isolated from frozen mycelial cells at −80 °C using Trizol reagent and following the procedure recommended by the manufacturer (Life Technologies). RNA concentration and purity were evaluated by measuring 260 nm and 280 nm absorbance with a Nanodrop spectrophotometer. The quality of total RNA was evaluated by agarose gel electrophoresis (Sambrook et al., 1989). For visualization of the fragments, the gel was exposed to Gel Documentation Systems. Total RNA samples were treated with DNAse (Fermentas) and posteriorly used for cDNA synthesis (Maxima ™ First Strand cDNA Synthesis kit - Fermentas), according to the manufacturer's instructions.

2.8. Analysis of gene expression by real-time PCR

cDNA was used after dilution (1/5) for real-time PCR analysis on the iQ5 (Bio-Rad) equipment. Each reaction consisted of 10 µL of SYBR Green (Thermo Scientific), 0.5 mM of each oligonucleotide (forward and reverse), 2 µL of template cDNA, and MiliQ water to a final volume of 20 µL. Expression of the gene encoding β-actin protein was used as the reference gene to normalize the total amount of cDNA present in each reaction (Steindorff et al., 2012). The reactions were performed in triplicate for each sample analyzed.

The reaction steps for real-time PCR were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, then followed by 60 °C dissociation curve up to 95 °C with successive increments of 0.5 °C for 10 s. Real-time PCR data demonstrated in all analyses the presence of only one peak in the melting curve, confirming that only one specific product was amplified.

2.9. Mathematical model and statistical analyzes

Quantification of gene expression was calculated according to the Livak method, $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). To facilitate the reading of expression level variations, the relative expression of the genes was presented in $\log_2$. To compare the relative expression levels of the genes analyzed in the experimental conditions with the conditions used as control, a simple variance analysis (ANOVA) was performed, which compares the tests with the control, using the program GraphPad Prism version 7.00. The significance level considered was 5%.

3. Results

3.1. General analysis of HSP proteins in Trichoderma asperellum

As Hsps proteins are still not well characterized in *Trichoderma* spp, we initially performed a search of the *T. asperellum* genome for the genes encoding sHsps, Hsps70, Hsps90 and Hsps100. These genes were chosen since there are some reports in the literature suggesting their participation during *Trichoderma* mycoparasitism (Marra et al., 2006; Seidl et al., 2009; Lorito et al., 2010; Steindorff et al., 2012). We observed that *T. asperellum* possesses four members of the sHsp family (IDs 53394, 271370, 44896 and 146718), twelve from the Hsp70 family (IDs 445543, 128301, 80694, 140277, 24918, 23336, 53053, 148983, 83736, 43441, 150656 and 188291), three from the Hsp100 family (IDs 59635, 48690 and 61928) and only one Hsp90 gene (ID 152105).

Table 2
T. asperellum Hsp proteins and their corresponding orthologues in other *Trichoderma* species. The numbers correspond to the percentage of similarity obtained by comparing the Hsp proteins of *T. asperellum* against the database of other fungi using the program BlatstP. The accession numbers for the proteins are shown in parentheses.

T. asperellum	T. atroviride	T. harzianum	T. reesei	T. virens
TaHsp26a (53394)	**84.8** (146119)	**77.2** (504208)	**71,4** (122251)	**78.1** (217094)
TaHsp26b (271370)	**95.2** (146319)	**71,6** (108885)	**65,5** (100314)	**72,2** (215292)
TaHsp26c (146718)	**94.8** (160834)	**65.2** (510360)	**70.0** (123565)	**88.1** (46820)
TaHsp70a (43441)	**97.5** (149341)	**96.8** (489504)	**94.5** (12362)	**96.8** (78895)
TaHsp70b (80694)	**91.7** (132465)	**90.2** (499175)	**89.9** (121926)	**90.2** (210885)
TaHsp70c (188291)	**94.6** (142538)	**92.8** (81081)	**92.4** (25648)	**92.3** (72131)
TaHsp90 (152105)	**95.0** (129493)	**94.0** (93459)	**93.0** (102206)	**94.0** (89650)
TaHsp104a (59635)	**96.9** (157172)	**95.2** (509199)	**94.5** (73724)	**94.8** (80583)
TaHsp104b (61928)	**94.4** (157453)	**93.5** (8037)	**93.2** (113088)	**93.7** (216898)

For this study, we analyzed the expression profiles of nine *T. asperellum* Hsp genes, namely *TaHsp26a, TaHsp26b, TaHsp26c, TaHsp70a, TaHsp70b, TaHsp70c, TaHsp90, TaHsp104a,* and *TaHsp104b*. We verified that the proteins encoded by these genes present homologs in other *Trichoderma* species, such as *T. atroviride*, *T. harzianum*, *T. reesei* and *T. virens* (Table 2). Furthermore, these proteins are predicted to localize to various cellular compartments: cytoplasm (TaHsp26a, TaHsp26b, TaHsp26c, TaHsp70b, TaHsp90 and TaHsp104b), mitochondria (TaHsp70a and TaHsp104a) and endoplasmic reticulum (TaHsp70c) (Supplementary data 2).

To evaluate the similarity between the Hsp proteins of the same family, a multiple sequence alignment was performed using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). We observed a high degree of amino acid conservation among representatives of the sHsp, Hsp70 and Hsp100 families, demonstrating that these proteins are highly conserved in *T. asperellum* (Fig. 1). We also verified a high similarity even at the N- and C-termini of sHsp proteins, regions that normally present very low levels of amino acid conservation.

We also analyzed the putative promoter region sequences of the nine *T. asperellum Hsp* genes, looking for sequences recognized by regulatory elements related to transcription activation as well as to stress responses (Supplementary data 3). Almost all *Hsp* genes showed motifs for TATA-box, CAAT-box and heat shock elements (HSE), except for *TaHsp70b* and *TaHsp90,* which did not show TATA-box sequences. *TaHsp70a*, *TaHsp70b*, *TaHsp70c*, *TaHsp104b* and mainly *TaHsp90* showed stress response elements (STRE). *TaHsp90* was also the only *Hsp* gene that contained a specific protein 1 (Sp1) motif.

3.2. Expression analysis of genes encoding Hsp proteins during T. asperellum self-recognition and mycoparasitism

To evaluate the relative expression of *Hsp* genes during mycoparasitism, we performed *T. asperellum* confrontation assays against itself (*T. asperellum* mycelium growing from both ends towards the center of the plate) and confrontation assays against *F. oxysporum* and *S. sclerotiorum*. We also analyzed expression levels of the *Hsp* genes during *T. asperellum* growth alone on plates at times that would correspond to pre-contact, contact and post-contact conditions (Figs. 2 and 3). We

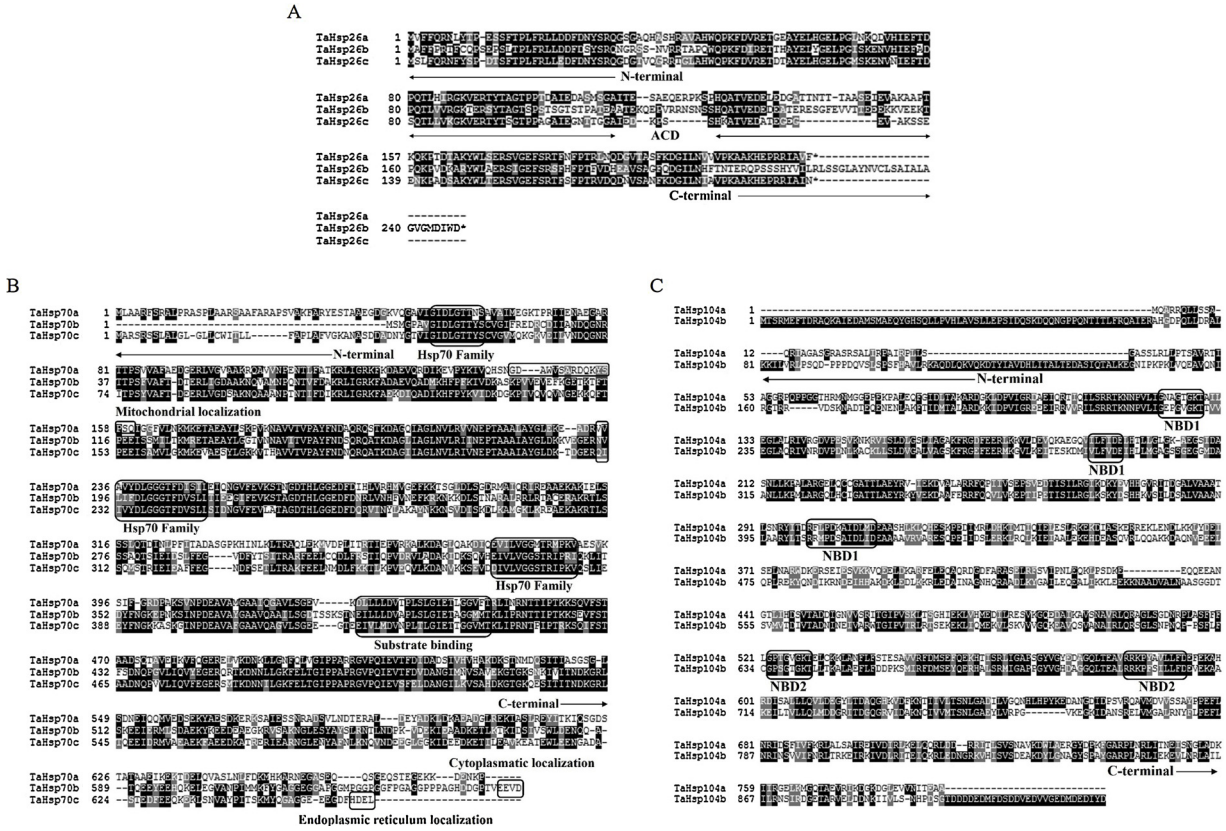

Fig. 1. Multiple alignment of amino acid sequences by Clustal Omega and visualization generated by the Boxshade program. **(A)-**TaHsp26a, TaHsp26b and TaHsp26c. ACD = Alpha Crystallin Domain. **(B)-** Hsp70a, Hsp70b and Hsp70c. Signature sequences are boxed. **(C)-** TaHsp104a and TaHsp104b. Signature sequences are boxed. NBD = Nucleotide Binding Domain. For both, the dark areas of the alignment indicate identical conserved amino acid residues; gray squares indicate changes in functionally conserved amino acids, and the dashes represent alignment gaps.

observed that most of the *Hsp* genes did not present significative variations in gene expression during the *T. asperellum* growth alone, with mRNAs corresponding to *TaHsp70* genes displaying the greatest abundance under this condition. However, we verified that the *TaHsp26c*, *TaHsp70b* and *TaHsp70c* genes were differentially expressed over time, indicating that these genes can be developmentally regulated in *T. asperellum*.

To better evaluate whether Hsp genes would be differentially expressed during *T. asperellum* interaction assays (either with itself or with phytopathogens), we analyzed the relative mRNA levels using the pre-contact condition as a reference **(**Figs. 4**A–I**).

Regarding the sHsp family, we observed that the *TaHsp26a* gene showed no significant expression during confrontations assays, whereas *TaHsp26b* and *TaHsp26c* were up-regulated in contact and post-contact conditions when grown against itself. These genes were also highly induced during post-contact both with *F. oxysporum* and *S. sclerotiorum* **(**Fig. 4**A, D and G**).

We observed that the *TaHsp70a* and *TaHsp70b* genes were induced under post-contact conditions against *S. sclerotiorum* but repressed under contact conditions against *F. oxysporum*. The *TaHsp70c* gene presented low levels in both confrontation tests against phytopathogens **(**Fig. 4**B, E and H**). Interestingly, we also noted that the *TaHsp70a*, *TaHsp70b* and *TaHsp70c* genes were induced in the post-contact condition when *T. asperellum* was grown in confrontation against itself.

The genes *TaHsp90*, *TaHsp104a* and *TaHsp104b* were significantly induced on contact and increased in the post-contact phase of *T. asperellum* against itself **(**Fig. 4**C, F and I**). When *T. asperellum* was confronted with *F. oxysporum* and *S. sclerotiorum* phytopathogens, the *TaHsp104a* and *TaHsp104b* genes were significantly upregulated in the post-contact phase. *TaHsp90* was not significantly induced in the confrontation of *T. asperellum* against *F. oxysporum* but was induced both during contact and post-contact with *S. sclerotiorum*.

3.3. Expression analysis of the genes encoding T. asperellum Hsp proteins during thermal stress

Thermal stress is the most recognized condition that positively regulates Hsp proteins. To further characterize *T. asperellum Hsp* genes, we evaluated the effects of prolonged exposure to heat shock on *Hsp* gene expression. For this, we submitted *T. asperellum* mycelia to thermal stress at 38 °C for 1, 2 or 4 h (Fig. 5).

T. asperellum exposed to thermal stress increased the amount of mRNA corresponding to all *TaHsp26* genes analyzed, as well as *TaHsp90*, *TaHsp104a* and *TaHsp104b*, with maximum levels observed after 1 h at 38 °C. Transcription levels decreased significantly after 4 h at high temperature, except for the *TaHsp26c* gene, whose mRNA levels remained high even after this period. The *TaHsp70a* and *TaHsp70b* genes were also upregulated by heat shock, while the *TaHsp70c* gene was not. Among *Hsp70* genes, *TaHsp70b* showed the greatest induction, with a significant increase in transcript levels after 1 h of treatment, which was maintained at a high level even after 4 h of heat stress.

A second heat shock test was performed with *T. asperellum*, which after growing for 48 h at 28 °C was subsequently exposed to temperatures of 4, 10 and 32 °C for 1 h (Fig. 6). Under cold shock at 4 °C, *TaHsp26a*, *TaHsp70c* and *TaHsp104a* genes were significantly induced. Meanwhile during the treatment at 10 °C, the *TaHsp70a* and *TaHsp104a* genes were the only ones upregulated. During heat shock at 32 °C almost all genes studied presented a large increase in the amount of mRNA, except for the *TaHsp70c* gene, which was downregulated.

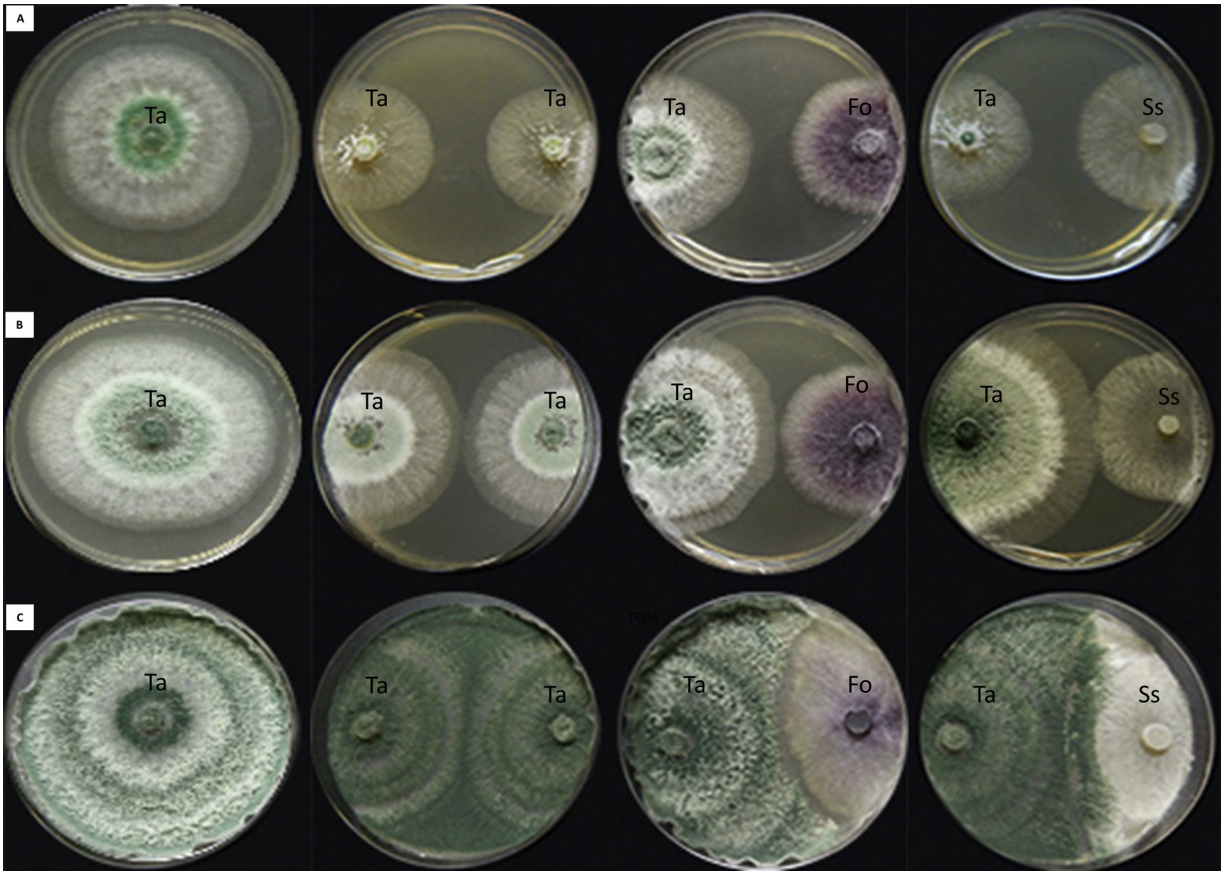

Fig. 2. Cultivation of *T. asperellum* growing alone, or in paired culture between *T. asperellum* x *T. asperellum*, *T. asperellum* x *F. oxysporum* and *T. asperellum* x *S. sclerotiorum*. Pre-contact **(A)**; Contact **(B)**; Post-contact **(C)**.

4. Discussion

The ability of some species of fungi to overcome environmental conditions has been previously related to Hsp, proteins that are highly conserved in eukaryotes and prokaryotes (Montero-Barrientos et al., 2010). Despite their importance for cellular maintenance, Hsp proteins are still poorly studied in *Trichoderma*, including *T. asperellum*; there are so far no articles presenting functional studies of *Trichoderma Hsp90* or *Hsp100* genes in the Pubmed database.

Hsp proteins have been associated with *Trichoderma* mycoparasitism

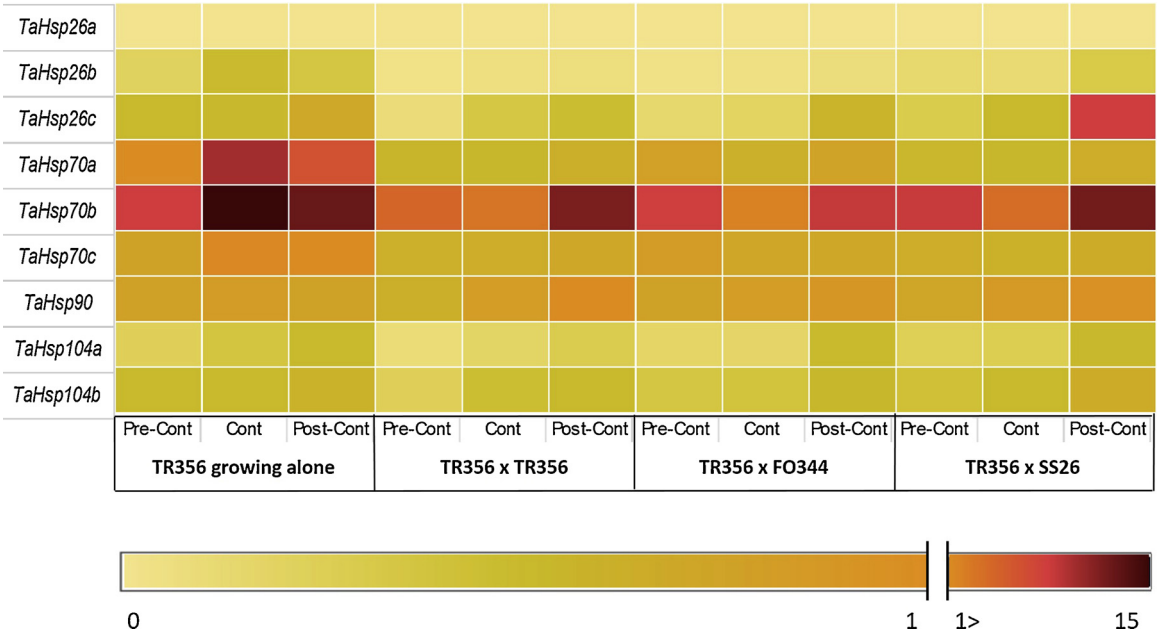

Fig. 3. Heat map of qPCR expression analyses of Hsp genes from *T. asperellum*. Data are represented as 2^{-}-ΔΔCT, and compared to the β-actin gene, used as a reference gene.

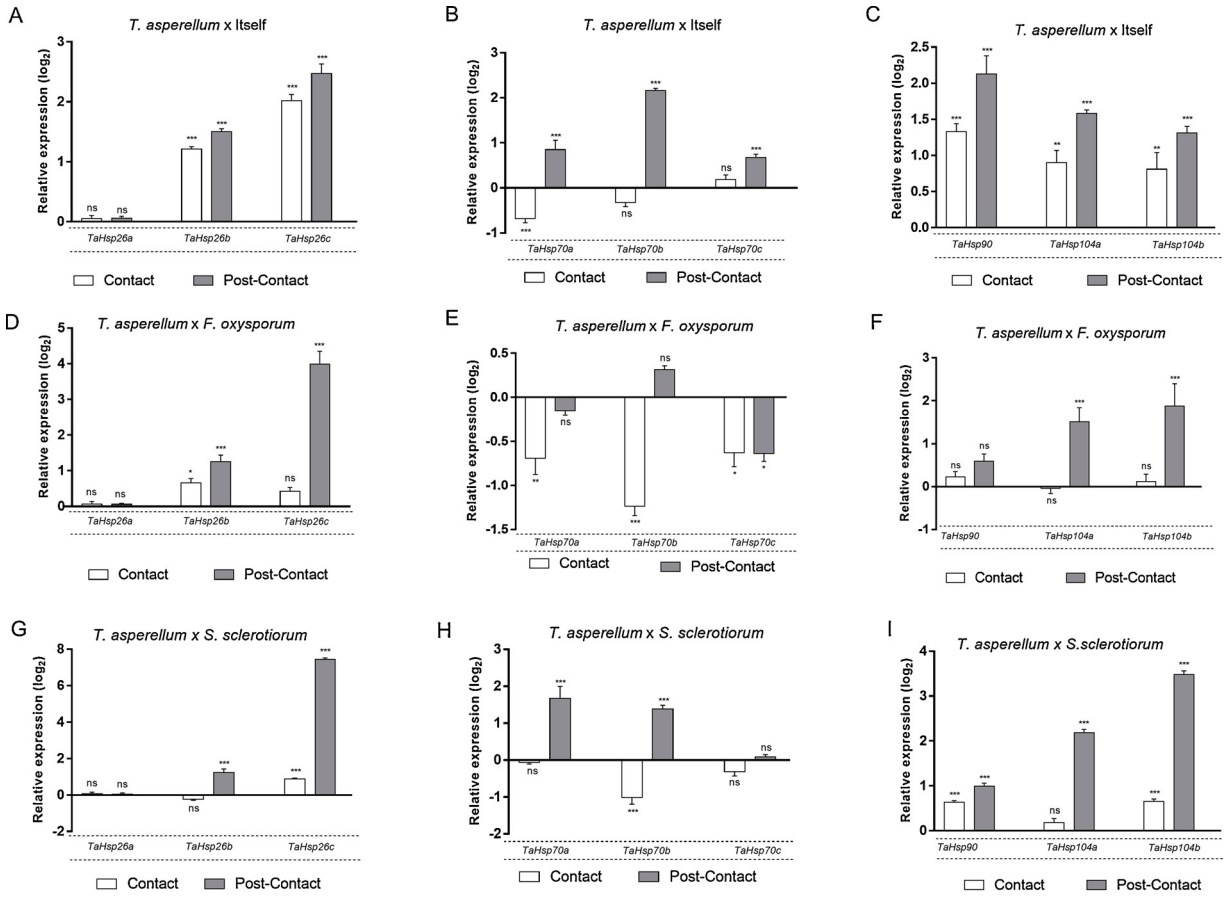

Fig. 4. Relative gene expression (log_2) of *TaHsp26a*, *TaHsp26b*, *TaHsp26c, TaHsp70a*, *TaHsp70b*, *TaHsp70c, TaHsp90*, *TaHsp104a*, and *TaHsp104b* genes of *T. asperellum* during contact and post-contact. **(A–C)**- Between identical colonies, **(D–F)** - *T. asperellum* in confrontation against *F. oxysporum*. **(G–I)**- *T. asperellum* in confrontation against *S. sclerotiorum*. mRNA levels during pre-contact condition were used as control condition. The data present the expression relative to the β-actin gene, used as reference gene. P value: (ns) 0.12, 0.0033 (*), 0.002 (**), < 0.0001 (***). α = 0.05.

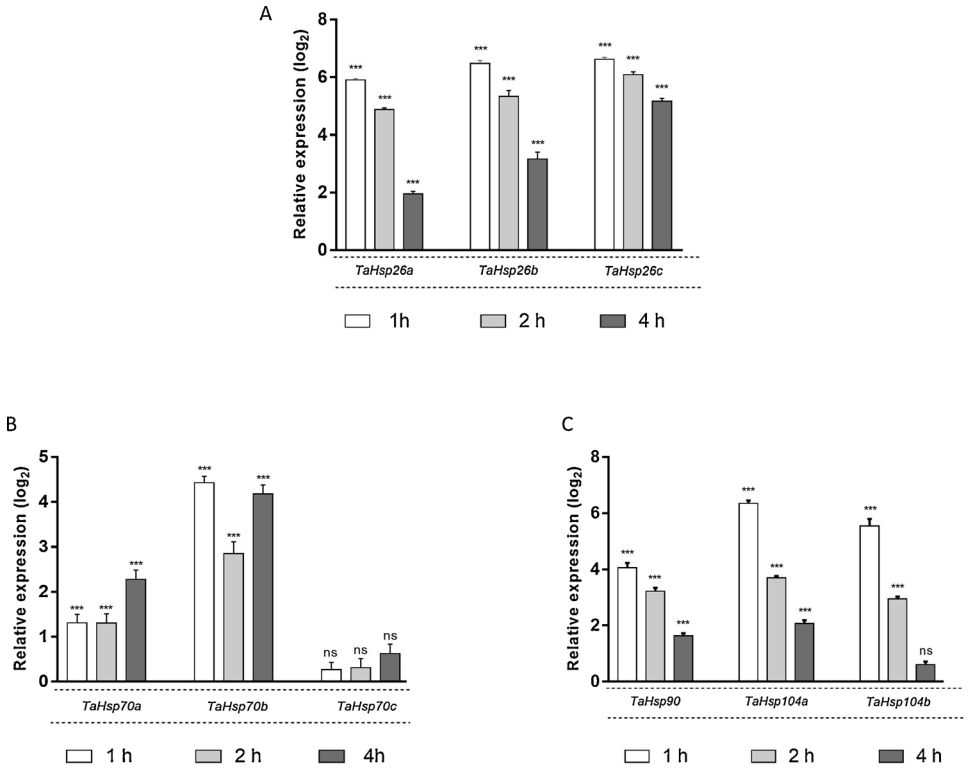

Fig. 5. Relative gene expression (log_2). **(A)**- *TaHsp26a*, *TaHsp26b* and *TaHsp26c;* **(B)**- *TaHsp70a*, *TaHsp70b* and *TaHsp7c*; **(C)**- *TaHsp90*, *TaHsp104a* and *TaHsp104b* of *T. asperellum* during heat stress treatment at 38 °C for 1, 2 and 4 h. Control temperature 28 °C. The data present expression relative to the β-actin gene, used as a reference gene. P value: (ns) 0.12, 0.0033 (*), 0.002 (**), < 0.0001 (***) α = 0.05.

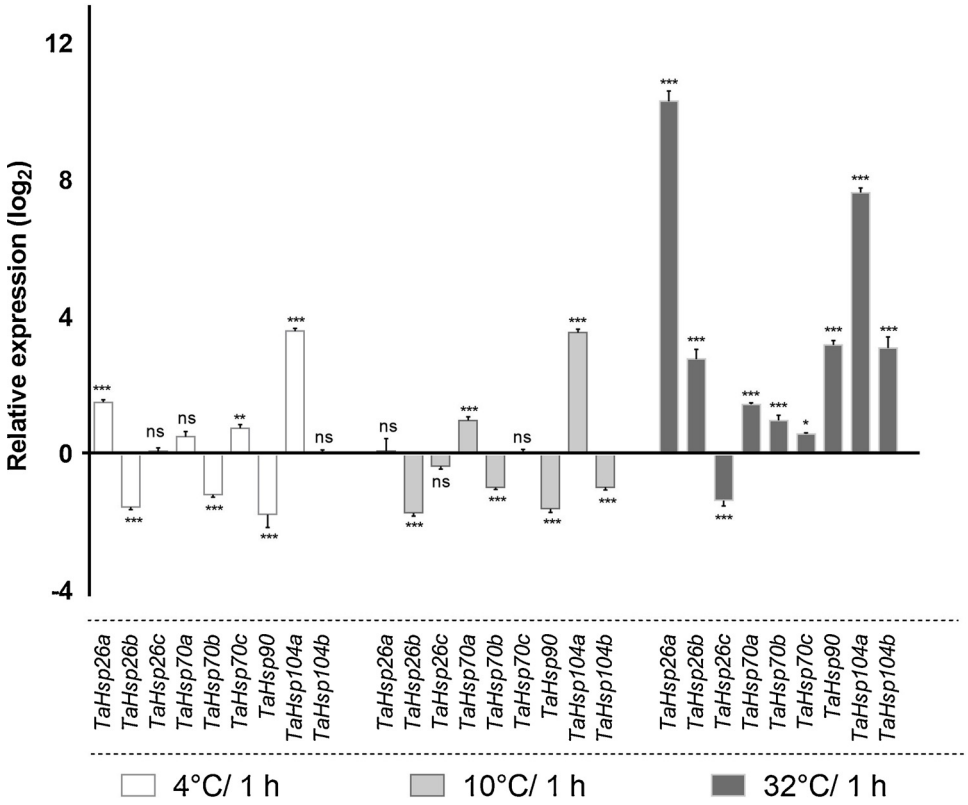

Fig. 6. Relative gene expression (log_2). *TaHsp26a*, *TaHsp26b*, *TaHsp26c, TaHsp70a*. *TaHsp70b*, *TaHsp70c*, *TaHsp90*, *TaHsp104a* and *TaHsp104b* of *T. asperellum* during heat stress treatment at 4 °C, 10 °C and 32 °C for 1 h. Control temperature 28 °C. The data present expression relative to the β-actin gene, used as a reference gene. P value: (ns) 0.12, 0.0033 (*), 0.002 (**), < 0.0001 (***) α = 0.05.

in some large-scale studies (Marra et al., 2006; Seidl et al., 2009; Lorito et al., 2010; Steindorff et al., 2012). However, detailed analysis has not yet been carried out. The work published by Seidl et al. (2009) evaluated the *T. atroviride* transcriptome response to the presence of *Botyris cinerea* and *Rhizoctonia solani*, two plant pathogens (as well as other experimental conditions). They performed this study by using EST sequencing and induction of *Hsp* genes by mycoparasitism, though the expression changes were not confirmed by northern blot or real-time PCR analysis.

In this work we analyzed the expression patterns of *Hsp* genes from *T. asperellum* during the self-recognition process, mycoparasitism and thermal stress. Of these three conditions, only the second was employed in the work of Seidl et al. (2009). Moreover, in the current study, mycoparasitism experiments were performed against two different phytopathogens (*S. sclerotiorum* and *F. oxysporum*). We also analyzed the expression of the *Hsp* genes during the interaction of *T. asperellum* with itself and observed that these genes were also important during self-recognition. Hsp have not previously been associated with this biological process. In addition, *Trichoderma* species are regularly exposed to temperature variations; for example, *T. asperellum*, one of the most abundant *Trichoderma* species found in Cerrado soils, can be exposed to temperatures reaching 40 °C (Lopes et al., 2012; Santos, 2010). Therefore, we also evaluated the expression profile of *Hsp* genes of *T. asperellum* in response to thermal stress, a condition not evaluated in previous studies. The fungus *T. asperellum* was chosen due to its high efficiency in the biological control of the phytopathogens such as *S. sclerotiorum* and *F. oxysporum*, (Geraldine et al., 2013).

The promoter region of almost all *T. asperellum Hsp* genes showed motifs for TATA-box, CAAT-box and heat shock elements (HSE). Interestingly, the presence of DNA sequences known as binding sites for stress-related transcriptional activators (STRE) was also found in the upstream region of the genes *TaHsp70a*, *TaHsp70b*, *TaHsp70c* and *TaHsp104b*. Except for the *TaHsp70c* gene, all others were induced by heat shock and during mycoparasitism.

After aligning the predicted amino acid sequences from the paralogous proteins TaHsp70a, TaHsp70b and TaHsp70c from *T. asperellum*, we noticed a high degree of similarity, with the presence of identical amino acids indicating conserved domains, which demonstrates the evolutionary importance of these proteins. In eukaryotes, Hsp70 proteins are found in all major compartments of the cell, usually represented by multiple Hsp70 homologues with distinct cellular functions (Craig et al., 1994). In fact, of the 12 genes encoding Hsp70 proteins in *T. asperellum*, eight are predicted to be cytoplasmic, two from endoplasmic reticulum and two from mitochondria.

Although the proteins from each Hsp family presented high amino acid similarity among themselves, we observed that their expression patterns were quite distinct. Hsp are involved in a variety of functions and are responsible for the maintenance of protein folding homeostasis related to folding, re-folding and degradation of damaged proteins (Parsell and Lindquist, 1993). Therefore, these different transcript levels could reflect different contributions of each gene to cellular homeostasis depending on the environmental conditions to which *T. asperellum* is exposed.

When analyzing *T. asperellum* growing alone over time, coinciding with the phases in which the mycelia are in pre-contact, contact and post-contact confrontation conditions, we observed changes in the expression of some of the *Hsp* genes studied (*TaHsp26c*, *TaHsp70b* and *TaHsp70c*). Differential expression of chaperones during different developmental stages has been reported in fungi. These cell differentiation stages consist mainly of the formation of vegetative or sexual spores and their germination, and vegetative growth phases (Plesofsky-Vig, 1996; Georg and Gomes, 2007a). It is known that Hsps act in fungal morphogenesis and play an important role in replication, transcription, post-transcriptional processing, translation, post-translational processes, and activation of signaling pathways (Tiwari et al., 2015).

Except for *TaHsp26a,* all other genes analyzed were induced in the post-contact condition when *T. asperellum* was cultured in a confrontation plate against itself. Additionally, *TaHsp26b*, *TaHsp26c*, *TaHsp90*, *TaHsp104a* and *TaHsp104b* were also induced during initial contact between *T. asperellum* hyphae, suggesting a role in the *T.*

asperellum self-recognition mechanism. If they were induced only because of competition for space or nutrients, these genes should also be expected to be induced during *T. asperellum* interaction with all phytopathogens, which is not what we observed. The expression of *T. asperellum* Hsp genes seems to be modulated by both the type of phytopathogen as well as *T. asperellum* interaction with itself, since we observed distinct expression patterns in each one of these conditions. It was previously reported that *T. atroviride* changes gene expression both in the presence of plant-pathogenic fungi and when interacting with itself (Reithner et al., 2011). Several proteins are important in self-recognition processes in fungi, especially cell wall proteins (Inbar and Chet, 1992; Gomes et al., 2015). However, to reach the extracellular compartment, proteins must be properly folded and translocated across membranes, a role performed by proteins from the Hsp family. The induction of these proteins during *T. asperellum* self-recognition could be important for the correct transport of proteins to the *T. asperellum* cell wall.

When we examined gene expression during mycoparasitism, it was observed that some *Hsp* genes were induced both by *S. sclerotiorum and F. oxysporum* (*TaHsp26b*, *TaHsp26c*, *TaHsp104a* and *TaHsp104b*), while others were not induced during interaction with either of the phytopathogens (*TaHsp26a* and *TaHsp70c*). Furthermore, we observed some genes induced only during confrontations against *S. sclerotiorum* (*TaHsp70a*, *TaHsp70b* and *TaHsp90*), indicating that the expression of *Hsp* genes during mycoparasitism seems to be modulated by the phytopathogen. In fact, it was observed that *Trichoderma* species can employ different mycoparasitism strategies against the same phytopathogen (Atanasova et al., 2013) and that the mycoparasitism process also depends on the pathogen type (Monteiro et al., 2010). During confrontation experiments against the phytopathogen *R. solani*, different sets of genes were up- or downregulated depending on the *Trichoderma* species analyzed. Per example, five chaperone/heat shock genes were upregulated in *Trichoderma virens* while in *Trichoderma reesei* no gene from this family was induced (Atanasova et al., 2013). Considering these data, it is natural that the *T. asperellum Hsp70* genes present a different expression profile during mycoparasitism compared to that observed in *T. atroviride* (Seidl et al., 2009), especially during interaction with different phytopathogens.

Seidl et al. (2009) observed by EST sequencing that some *Hsp* genes present high mRNA abundance in cDNA libraries constructed from RNA extracted from *T. atroviridae* mycelia during mycoparasitism against *R. solani*. In *T. asperellum,* the *TaHsp26b*, *TaHsp26c*, *TaHsp70a*, *TaHsp70b*, *TaHsp90, TaHsp104a* and *TaHsp104b* genes appear to be differentially expressed during mycoparasitism, being induced in the final phase, during post-contact and/or contact with *S. sclerotiorum*. Studies on hydrolytic enzymes produced by *Trichoderma* species during mycoparasitism show that genes encoding these enzymes were also induced mainly after contact with phytopathogenic fungi (Qualhato, 2013).

To evaluate whether *Hsp* genes are expressed during temperature oscillations we analyzed their transcript levels during heat and cold shock. When we analyzed heat stress over time we observed that the induction pattern of the *TaHsp26a, TaHsp26b, TaHsp26c TaHsp90*, *TaHsp104a* and *TaHsp104b* genes were quite similar, indicating that the heat shock induction is transient. To maintain their functioning, cells need to respond rapidly and intensely to the denaturation of proteins provoked by the increase in temperature. This expressive synthesis of Hsps cannot be maintained indeterminately, so the induction of *Hsps* in response to heat stress is usually transient (Georg and Gomes, 2007a; Pugliese et al., 2008).

Concerning thermal stress, the *TaHsp26a* gene should be highlighted, since it was the only one that was highly induced by heat shock at 38 °C but was not expressed during the growth of *T. asperellum* alone, nor during the confrontation assays, either with itself or with phytopathogens. Also, we did not observe significant differences in the promoter region of the three *TaHsp26* genes that justify this difference in *TaHsp26a* expression. In addition, *TaHsp26a* was upregulated both during cold shock at 4 °C and mild-heat shock at 32 °C. Despite being upregulated by severe heat stress (41 °C), the *T. virens hsp23* gene was more induced by low (4 and 10 °C) than high temperatures. In contrast, the *hsp23* gene of *T. harzianum* was highly induced by thermal shock at 41 °C and no expression was observed at lower temperatures (Montero-Barrientos et al., 2007). In this sense, it is possible to observe that the expression of *Hsp* genes is distinct for different species of *Trichoderma*, even in response to thermal stress, a condition expected to cause induction of these genes.

The *TaHsp90* gene showed abundant mRNA levels during *T. asperellum* growth and was upregulated during self-recognition, mycoparasitism against *S. sclerotiorum,* and thermal stress. In most organisms and cell types, Hsp90 proteins are already abundant prior to cellular stress and are typically induced only a few-fold. It has been estimated that Hsp90 accounts approximately for 1% of the total soluble cytosolic protein in unstressed cells, making it one of the most abundant proteins in the cell (Lai et al., 1984; Schopf et al., 2017).

Another gene that called attention is *TaHsp104a*. We observed that this gene was upregulated in all conditions analyzed, with higher levels of induction observed during thermal stress, both during heat and cold shock. In *T. atroviride*, *hsp104* gene expression was observed during mycoparasitism against *R. solani* (Seidl et al., 2009). In *T. asperellum*, the *TaHsp104a* gene appears to be part of the general stress response. Nevertheless, Hsp100 proteins do not act alone, requiring interaction with sHsp and Hsp70 proteins to disaggregate protein complexes (Mogk et al., 2003).

TaHsp70 genes were those that presented the most abundant mRNA levels during *T. asperellum* growth, with *TaHsp70a* and *TaHsp70b* induced in response to heat stress. Ryan et al. (1991) have suggested that the amount of synthesized Hsp70 is dependent on the severity of the stress to which the organism is subjected, and the existing Hsp70 cellular levels before the stress condition. The importance of Hsp proteins in response to heat stress is well documented, although not all Hsp is induced by high temperatures. For instance, the aquatic fungus *Blastocladiella emersonii* possesses ten *Hsp70* genes and only seven of them are significantly induced by heat shock (Georg and Gomes, 2007b). In fact, the *TaHsp70c* gene seems to be constitutively expressed, since it was not induced by heat stress nor by confrontation with phytopathogens.

It is known that the synthesis of Hsp70 proteins in heat-exposed cells is reduced when the same cells are subsequently exposed to a second heat stress (Ryan et al., 1991). This adaptive condition was called acquired thermotolerance and is an important response of environmental thermal regulation (Gerner and Scheneider, 1975). This acquisition of thermotolerance may explain the ability of some *Trichoderma* strains to overcome adverse environmental conditions, facilitating their presence in different geographic locations (Hermosa et al., 2004; Montero-Barrientos, 2008).

We observed that *TaHsp26a*, *TaHsp70a*, *TaHsp70c* and *TaHsp104a* were induced in response to cold shock. Several factors may interfere with the performance of *Trichoderma* as a biocontrol agent, and among these is low temperature (Kredics et al., 2003). In fact, when 360 isolates of *Trichoderma* spp. were assessed in relation to tolerance to cold stress, only 14 grew well at 5 °C, corresponding to *T. aureoviride*, *T. harzianum* and *T. viride* species (Antal et al., 2000). Although Hsp proteins are more frequently induced in response to heat shock, their induction is also observed in response to low temperature stress (Montero-Barrientos et al., 2007; Wang et al., 2017).

Acknowledgment

The authors are grateful to CAPES and CNPq for financial support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:https://doi.org/10.1016/j.micres.2019.126296.

References

Altschul, S.F., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Antal, Z., et al., 2000. Colony growth, in vitro antagonism and secretion of extracellular enzymes in cold-tolerant strains of *Trichoderma* species. Mycol. Res. 104, 545–549. https://doi.org/10.1017/S0953756299001653.

Atanasova, L., et al., 2013. Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism. BMC Genomics 14, 121. https://doi.org/10.1186/1471-2164-14-121.

Blum, T., et al., 2009. MultiLoc2: integrating phylogeny and Gene Ontology terms improves subcellular protein localization prediction. BMC Bioinformatics 10, 274. https://doi.org/10.1186/1471-2105-10-274.

Craig, E.A., et al., 1994. Cytosolic hsp70s of saccharomyces cerevisiae: roles in protein synthesis, protein translocation, proteolysis, and regulation. In: Morimoto, R., Tissieres, A., Georgopoulos, C. (Eds.), The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor, New York, pp. 31–52.

Druzhinina, I.S., et al., 2018. Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus *Trichoderma* from its plant-associated hosts. PLoS Genet. 14, e1007322. https://doi.org/10.1371/journal.pgen.1007322.

Fuller, K.J., et al., 1994. Cancer and the heat shock response. Eur. J. Cancer 30, 84–91. https://doi.org/10.1016/0959-8049(94)00362-9.

Georg, R.C., Gomes, S.L., 2007a. Transcriptome analysis in the aquatic fungus *Blastocladiella emersonii* in response to heat shock and cadmium. Eukaryot. Cell 6, 1053–1062. https://doi.org/10.1128/EC.00053-07.

Georg, R.C., Gomes, S.L., 2007b. Comparative expression analysis of members of the Hsp70 family in the chytridiomycete *Blastocladiella emersonii*. Gene. 386, 24–34. https://doi.org/10.1016/j.gene.2006.07.033.

Geraldine, A.M., et al., 2013. Cell wall-degrading enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma* spp. Biol. Control. 67, 308–316. https://doi.org/10.1016/j.biocontrol.2013.09.013.

Gerner, E.W., Scheneider, M.J., 1975. Induced thermal resistance in HeLa cells. Nature. 256, 500–502. https://doi.org/10.1038/256500a0.

Gomes, E.V., et al., 2015. The cerato-platanin protein Epl-1 from *Trichoderma harzianum* is involved in mycoparasitism, plant resistance induction and self cell wall protection. Sci. Rep. 5, 17998. https://doi.org/10.1038/srep17998Als.

Hermosa, M.R., et al., 2004. Genetic diversity shown in *Trichoderma* biocontrol isolates. Mycol. Res. 108, 897–906. https://doi.org/10.1017/S0953756204000358.

Inbar, J., Chet, I., 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. J. Bacteriol. 174, 1055–1059. https://doi.org/10.1128/jb.174.3.1055-1059.1992.

Jäättelä, M., Wissing, D., 1992. Emerging role of heat shock proteins in biology and medicine. Ann. Med. 24, 249–258. https://doi.org/10.3109/07853899209149952.

Jourdier, E., et al., 2017. Proximity ligation scaffolding and comparison of two *Trichoderma reesei* strains genomes. Biotechnol. Biofuels 10, 151. https://doi.org/10.1186/s13068-017-0837-6.

Koike, H., et al., 2013. Comparative genomics analysis of *Trichoderma reesei* strains. Ind. Biotech. 9, 352–367. https://doi.org/10.1089/ind.2013.0015.

Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics. 23, 1289–1291. https://doi.org/10.1093/bioinformatics/btm091.

Kredics, L., et al., 2003. Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. Food Technol. Biotechnol. 41, 37–42.

Lai, B.T., et al., 1984. Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. Mol. Cell Biol. 4, 2802–2810. https://doi.org/10.1128/MCB.4.12.2802.

Lamoth, F., et al., 2015. Histone deacetylase inhibition as an alternative strategy against invasive aspergillosis. Front. Microbiol. 6, 96. https://doi.org/10.3389/fmicb.2015.00096.

Lima, L.H.C., De Marco, J.L., Felix, C.R., 1998. Enzimas hidrolíticas envolvidas no controle biológico por micoparasitismo. In: Melo, I.S., Azevedo, J.L. (Eds.), Controle biológico. Embrapa Meio Ambiente, Jaguariúna, SP.

Lindquist, S., 1986. The heat-shock response. Ann Rev Biochem. 55, 1151–1191. https://doi.org/10.1146/annurev.bi.55.070186.005443.

Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Annu. Rev. Genet. 22 (1), 631–677. https://doi.org/10.1146/annurev.ge.22.120188.003215.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) Method. Methods. 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lorito, M., et al., 2010. Translational research on *Trichoderma*: from' omics to the field. Annual Reviews of Phytopathology. 48, 395–417. https://doi.org/10.1146/annurev-phyto-073009-114314.

Lopes, F.A., et al., 2012. Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian Cerrado, and potential antagonism against *Sclerotinia sclerotiorum*. Fungal Biol. 116, 815–824. https://doi.org/10.1016/j.funbio.2012.04.015.

Marra, R., et al., 2006. Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. Curr. Genet. 50, 307–321. https://doi.org/10.1007/s00294-006-0091-0.

Mogk, A., et al., 2003. Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. Mol. Microbiol. 50, 585–595. https://doi.org/10.1046/j.1365-2958.2003.03710.x.

Monteiro, V.N., et al., 2010. New Insights in *Trichoderma harzianum* antagonism of fungal plant pathogens by secreted protein analysis. Curr. Microbiol. 61, 298–305. https://doi.org/10.1007/s00284-010-9611-8.

Montero-Barrientos, M., et al., 2007. The heterologous overexpression of *hsp23*, a small heat-shock protein gene from *Trichoderma virens*, confers thermotolerance to *T. Harzianum*. Curr. Genet. 52, 45–53. https://doi.org/10.1007/s00294-007-0140-3.

Montero-Barrientos, M., et al., 2008. Overexpression of a *Trichoderma* HSP70 gene increases fungal resistance to heat and other abiotic stresses. Fungal Genet. Biol. 45, 1506–1513. https://doi.org/10.1016/j.fgb.2008.09.003.

Montero-Barrientos, M., et al., 2010. Transgenic expression of the *Trichoderma harzianum hsp70* gene increases *Arabidopsis* resistance to heat and other abiotic stresses. J. Plant Physiol. 167, 659–665. https://doi.org/10.1016/j.jplph.2009.11.012.

Parsell, D.A., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27, 437–496. https://doi.org/10.1146/annurev.ge.27.120193.002253.

Plesofsky-Vig, N., Brambl, R., 1993. Heat shock proteins in fungi. In: Jennings, D.H. (Ed.), Stress Tolerance of Fungi. Marcel Dekker, New York, pp. 45–68.

Plesofsky-Vig, N., 1996. The heat shock proteins and the stress response. Biochem. Mol. Biol. 3, 171–190. https://doi.org/10.1007/978-3-662-10367-8_7.

Pugliese, L., et al., 2008. Expression of genes encoding cytosolic and endoplasmic reticulum HSP90 proteins in the aquatic fungus *Blastocladiella emersonii*. Gene 411, 59–68. https://doi.org/10.1016/j.gene.2008.01.005.

Qualhato, T.F., 2013. Avaliação do perfil enzimático e expressão gênica de cinco espécies de Trichoderma e potencial antagônico contra *Fusarium solani*, *Rhizoctonia solani* e *Sclerotinia sclerotiorum*. Goiânia. Dissertação [Mestrado em Biologia]. Universidade Federal de Goiás.

Reithner, B., et al., 2011. Identification of mycoparasitism-related genes in *Trichoderma atroviride*. Appl. Environ. Microbiol. 77, 4361–4370. https://doi.org/10.1128/AEM.00129-11.

Ryan, A.J., Gisolfi, C.V., Moseley, P.L., 1991. Synthesis of 70K stress protein by human leukocytes: effect of exercise in the heat. J. Appl. Physiol. 70, 466–471. https://doi.org/10.1152/jappl.1991.70.1.466.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Santos, F.B.N., 2010. . Padrões de distribuições espaciais e temporais de temperatura de superfície no bioma Cerrado: Uma análise integrada a partir de dados orbitais de resolução moderada. Goiânia. Tese [Doutorado em Ciências Ambientais]. Universidade Federal de Goiás.

Schmoll, M., et al., 2016. The genomes of three uneven siblings: footprints of the lifestyles of three *Trichoderma* species. Microbiol. Mol. Biol. Rev. 80, 205–327. https://doi.org/10.1128/MMBR.00040-15.

Schopf, F.H., et al., 2017. The Hsp90 chaperone machinery. Nat. Rev. Mol. Cell Biol. 18, 345–360. https://doi.org/10.1038/nrm.2017.20.

Seidl, V., et al., 2009. Transcriptomic response of the mycoparasitic fungus Trichoderma atroviride to the presence of a fungal prey. MC Genomics. 10, 567. https://doi.org/10.1186/1471-2164-10-567.

Sievers, F., et al., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Biol. 7, 539. https://doi.org/10.1038/msb.2011.75.

Steindorff, A.S., et al., 2012. *Trichoderma harzianum* expressed sequence tags for identification of genes with putative roles in mycoparasitism against *Fusarium solani*. Biol. Control. 61, 134–140. https://doi.org/10.1016/j.biocontrol.2012.01.014.

Steindorff, A.S., et al., 2014. Identification of mycoparasitism-related genes against the phytopathogen *Sclerotinia sclerotiorum* through transcriptome and expression profile analysis in Trichoderma harzianum. BMC Genomics 15, 204. https://doi.org/10.1186/1471-2164-15-204.

Tereshina, V.M., 2005. Thermotolerance in fungi: the role of heat shock proteins and trehalose. Microbiology. 74, 247–257. https://doi.org/10.1007/s11021-005-0059-y.

Thrane, C., Tronsmo, A., Jensen, D.F., 1997. Endo-β-1,3-glucanase and cellulose from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic Pythium spp. Eur. J. Plant Pathol. 103, 331–344. https://doi.org/10.1023/A:1008679319544.

Tiwari, S., Thakur, R., Shankar, J., 2015. Role of heat-shock proteins in cellular function and in the biology of fungi. Biotechnol. Res. Int. 2015, 132635. https://doi.org/10.1155/2015/132635.

Untergasser, A., et al., 2012. Primer3 - new capabilities and interfaces. Nucleic Acids Res. 40, e115. https://doi.org/10.1093/nar/gks596.

Vierling, E., 1991. The roles of heat shock proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 579–620. https://doi.org/10.1146/annurev.pp.42.060191.003051.

Wang, D., et al., 2017. De novo transcriptome sequencing of *Isaria cateniannulata* and comparative analysis of gene expression in response to heat and cold stresses. PLoS One 12, e0186040. https://doi.org/10.1371/journal.pone.0186040.