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Research Article

Expression and localization of tubulin isotypes and its mRNAs during *Thecaphora frezii* developments

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Abstract

Thecaphora frezii is a phytopathogenic fungus that infects *Arachys hypogaea* L. and produces peanut smut. It has three ontological stages teliospores, basidiospores, and hyphae. Microtubules are cellular structures that participate in various important cellular processes. In this work, we analyzed the presence and location of α-tubulin isotypes and enzymes that participate in tyrosination–detyrosination in the three stages of *T. frezii*. Although both tyrosinated and detyrosinated tubulin seem to be associated with a membrane fraction component that gives it a similar behavior to integral proteins, in the soluble cytosolic fraction, only detyrosinated tubulin was detected, not tyrosinated tubulin. The presence of α-tubulin was not detected using the monoclonal antibody DM1A as neither acetylated tubulin. The RNA-Seq analysis showed the presence of α, β, and γ-tubulins and the genes that codes for tyrosine–tubulin ligase and cytosolic carboxypeptidase 1, enzymes that are involved in post-translational modification processes. These sequences showed a high percentage of identity and homology with *Ustilago maydis*, *Thecaphora thlaspeos*, and *Anthracocystis flocculosa*. This is the first report for tubulins subpopulations and the cellular distribution in *T. frezii*, which together with the data obtained by RNA-Seq contribute to the knowledge of the pathogen, which will allow the development of control strategies

Keywords: cytosqueleton, detyrosinated tubulin, immunostaining, mRNA sequence, peanut smut, tyrosinated tubulin

Introduction

Thecaphora frezii is a phytopathogenic fungus belonging to the ustilaginomycetes class; it infects *Arachys hypogaea* L, producing peanut smut disease (Carranza and Lindquist 1962), which leads to significant economic crop loss. During the biological cycle of the fungus, three structures are distinguished: teliospores, basidiospores, and the hyphae. When gynophore penetrates the soil, tissue is colonized by infective hyphae, which replace tissue cells with reddish-brown teliospores (Marinelli et al. 2008). Genetic and biochemical modifications occur in this transition from one state to another, where microtubules are also involved.

Microtubules are important components of the cytoskeleton of eukaryotic cells, they are essential for many processes, spindle assembly and function, chromosome segregation, organelle movement, transportation of proteins, and maintenance of growth polarity (Westermann and Weber 2003).

In most fungi, microtubules are part of the mitotic spindle and as tracks in the cytoplasm (Xiang and Plamann 2003). In filamentous fungi, the microtubules are important for the rapid growth of hyphae (Horio 2007), nuclear migration, transport of organelles, protein, and RNA, involved in pathogenic development, being essentially for long-range transport (Fuchs et al. 2005, Shield 2014).

Microtubules are assembled from α/β tubulin heterodimers, where γ-tubulin may also participate (Shield 2014). The γ-tubulin isoform was first discovered in *Aspergillus nidulans* and plays an essential role in the initiation of microtubule assembly, as it is necessary for the nucleation of microtubule polymerization (Xiang and Plamann 2003). They have specific functions according to the stage of development of the cell; Fuchs et al. (2005) showed that microtubules in *Ustilago maydis* are essential for nuclear migration, therefore, they are essential in long-distance hyphal growth, and they are dispensable for the polarized growth during the morphological transition of yeast-like cells. The dynamic state of microtubules is regulated by their ability to bind specific proteins on their surface, microtubule-associated proteins (MAPs; Shield 2014).

Tubulins are subject to a large range of post-translational modifications (PTMs), such as detyrosination/tyrosination, acetylation, phosphorylation, polyglutamylation, and polyglycylation. These PTMs affect the dynamics of the microtubules, their organization, and interaction with other cellular components as MAPs or motor proteins (Janke and Magiera 2020). There are specific enzymes that participate in these post-translational processes, acetyl-transferase α-TAT1 is one of the enzymes that catalyzes the acetylation of K40 in α-tubulin, and the reverse reaction is catalyzed by the deacetylases Sirtuin 2 (SIRT2) or HDAC6. The formation of detyrosinated tubulin (Glu-Tub) is mediated by carboxypeptidase (detyrosination), and the removed tyrosine residue can be religated by tubulin–tyrosine ligase (TTL; Song and Brady 2015, Wloga et al. 2017).

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At present, there are no data on the distribution, location, and PTMs of *T. frezii* α-tubulin and considering the importance of microtubules and the changes that this fungus suffers during its development; we characterized the presence and subcellular location of different PTMs on α-tubulin in different stages of development of *T. frezii*. On the other hand, using RNA-Seq, we identify the presence of tubulin genes and those that codify for enzymes involved in their PTMs in the three developmental structures.

Detyrosinated tubulin was located in both the cytosolic and membrane fractions in teliospores, basidiospores and hyphae, while tyrosinated tubulin was only present in the membrane fraction, interacting with some component of it. The sequence of enzymes tyrosine-tubulin ligase and cytosolic carboxypeptidase 1 were deduced and showed high percentage of identity and homology with *Ustilago maydis*, *Antracocystis flocculosa* and *Thecaphora thlaspeos*.

Materials and methods

Materials

Triton X-100, TritonX-114, 4-chloro naphthol, phenyl methyl sulfonyl fluoride (PMSF), 1, 4-dithiothreitol (DTT), ethylene diamine tetra acetic acid (EDTA), glycol ether diamine tetra acetic acid (EGTA), lysing enzymes from *Trichoderma harzianum*, PIPES, magnesium sulphate, sodium citrate, bovine serum albumin, sodium citrate, hydrogen peroxide, sorbitol, and glycerol were from Sigma Aldrich. Acetone and formaldehyde were from Cicarelli. Nitrocellulose membrane was from Bio-Rad. Rabbit polyclonal antidetyrosinated tubulin antibody was a gift from Dr. Carlos Arce (Dpto. Biological Chemistry of the National University of Córdoba). Mouse monoclonal anti-α-tubulin (clone DM1A), mouse monoclonal antiacetylated tubulin (6–11B-1), mouse monoclonal antityrosinated tubulin (TUB1A2) primary antibodies, and antimouse IgG and antirabbit IgG peroxidase or FITC conjugate secondary antibodies, were purchased from Sigma Aldrich. Potato broth was from Neogen and potato dextrose agar (PDA) from the Britania.

Culture conditions

Teliospores of *T. frezii* were obtained from carbonaceous peanut pods previously disinfected with 0.5% NaOCl (v/v) with agitation for 15 min and rinsed with sterile water. Teliospores were then disinfected in 1% NaOCl (v/v) for 5 min, rinsed twice with sterile distilled water, and resuspended in physiological solution. One part was seeded on PDA and incubated at 26°C in the dark until germination. Once the hyphae developed, they were maintained by subcultures in the same medium. Basidiospores were obtained from a hyphal culture transferred to agar/water (1.5% w/v) after 10 days of incubation at 26°C in dark; under this experimental condition around 90% of the hyphae were transformed into basidiospores. The hyphae cultures were grown to logarithmic phase in potato broth with shaking at 26°C (Soria et al. 2021).

Protein extraction

For proteins extraction, hyphae cultures of 24 h, basidiospores, and teliospores previously disinfected were harvested by centrifugation at 2000 × *g* for 5 min. Cell pellets were washed twice with physiologic solution and resuspended in cold lysis buffer (phosphate buffer 50 mM, pH: 7, EDTA 5 mM, DTT 5 mM, PMSF 1 mM, and glycerol 20% v/v) and disrupted in a mortar and pestle using liquid nitrogen. The homogenate was centrifuged at 20 000 × *g* for 40 min at 4°C; the supernatant from this first treatment contains cytosolic proteins. Then, pellets were resuspended in TBS (10 mM Tris-HCl pH: 7.4, 150 mM NaCl) containing 1% (w/v) Triton X-114 to obtain membrane proteins. The solution was held 60 min at 4°C and then centrifuged for 15 min at 20 000 × *g* to remove insoluble materials. The supernatant was incubated at 35°C for 5 min and then centrifuged at 3000 × *g* for 5 min. The upper aqueous phase (AP) and the lower detergent rich phases (DP) were carefully separated.

All proteins were concentrated by precipitation with acetone and dissolved in phosphate buffered saline (PBS) with 1% SDS before quantification. Protein concentration was measured according to Bradford (1976) using bovine serum albumin as the standard.

Identification of tubulin by dot blot

Tubulin from teliospore, basidiospore, and hyphae of *T. frezii* were compared by dot blot hybridization. Proteins (75 μg) from cytosolic and membrane fractions (AP and DP) of *T. frezii* were applied directly on individual to nitrocellulose membrane, fixated in methanol acetic at room temperature for 10 min, and washed with water twice and with PBS three times. Afterwards, membrane was blocked with 5% nonfat milk in PBS by 30 min and incubated with primary antibody overnight at 4°C. Mouse monoclonal antibody clone DM1A (dilution 1/1000) specific to total α-tubulin, mouse monoclonal antibody 6–11B-1 (dilution 1/1000) for acetylated tubulin, mouse monoclonal antibody TUB1A2 (dilution 1/1000) specific for tyrosinated tubulin (Tyr-Tub), and rabbit polyclonal antibody (dilution 1/500) for detyrosinated tubulin (Glu-Tub) were used. Then, membranes were washed five times with 0.1% triton X-100 in PBS and incubated for 2 hours at 37°C with the secondary antibody labeled with horseradish peroxidase, diluted 1:250 in 5% skim milk in PBS (antimouse IgG or antirabbit IgG by Glu-Tub). Membranes were washed again for three times with 0.1% triton X-100 in PBS followed by three washes with TBS and inserted into a plate loaded with substrate solution (4-chloro naphthol) until color develop. The reaction was stopped by addition of water. A rat brain protein extract was used as a positive sample control for tubulins immunodetection.

Tubulin membrane interaction

In order to inquire about the nature of the presence of tubulin in membranes in teliospores, we also followed the protocol described by Beltramo et al. (1994). They show that tubulins present in brain membranes are associated with some other component and that they can be separated from integral membrane proteins by alkaline treatment. Thus, membranes of teliospores were resuspended by homogenization in TBS and 100 mM Na_2CO_3 pH: 12 were added. The suspension was incubated for 30 min at 4°C and then centrifuged at 20 000 × *g* for 30 min. Next, the pellet was extracted with triton X-114 as described above, after heating an upper AP and the lower DP were obtained. Then, all these protein extracts were fixed on nitrocellulose membranes and incubated with a rabbit polyclonal antibody (dilution 1/500) against Glu-Tub, as described in the dot blot test.

Indirect immunofluorescence

For microtubule immunodetection, the samples were treated according to the protocol of Banuett and Herskowitz (2002) with some variations. Hyphae and basidiospores fresh culture were fixed with PEM buffer (50 mM PIPES buffer, pH: 6.7, 25 mM EGTA, pH 7.0, and 50 mM MgSO4) with 3.7% v/v formaldehyde, for 40 min at RT and centrifuged to 3000 × *g* for 5 min. The pellet was wash three times with the same buffer without formaldehyde. These

fixed cells were seeded onto poly-D-lysine-coated coverslips and digested for 1 hour at RT with a cell wall lytic enzyme solution (2 mg/ml lysing enzymes of *T. harzianum* in 1 M sorbitol, 50 mM sodium citrate pH: 5.7, and 5 mM EGTA). After the incubation time it was washed three times with PEM buffer and incubated with one drop of permeabilization buffer (100 mM PIPES buffer, pH: 6.7, 25 mM EGTA, pH: 7.0, and 0.1% triton X-100) for 5 min and washed twice with PEM buffer. The samples were blocked for 30 min in PBS 1% BSA and subsequently incubated with the primary anti Glu-Tub antibody, Tyr-Tub antibody, and α-tubulin DM1A clone antibody (1/200 in PBS 1% BSA), overnight at 4°C in humidified chamber. After washing, the samples were incubated for 1 hour in the corresponding secondary antibody FITC conjugate (dissolved 1/250 PBS 1% BSA) washed with PBS and instantly processed for fluorescence microscopy.

Microscope slides were examined with an Axio Observer.D1 Carl Zeiss microscope adapted with an axiocam 506 mono Camera. Images were processed with Zen 2 lite program.

RNA extraction

Total RNA from each stages of *T. frezii* (three independent cultures were pooled for each one) was extracted using TRIzol (Invitrogen, USA) following the manufacturer's recommendations. gDNA was removed by column digestion with DNase (Qiagen, USA) at twice the concentration recommended by the manufacturer.

The potential degradation and contamination of the RNA was monitored by means of a 1.5% (w / v) agarose gel and the purity of the RNA was confirmed using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured with the Qubit RNA Assay Kit and the Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was re-evaluated with the RNA Nano 6000 Assay Kit and the Agilent Bioanalyzer.

Library and RNA-seq preparation

RNA from all three stages of *T. frezii* was used to generate the cDNA library. Sequencing libraries were created using the NEB Next Ultra RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's instructions. Briefly, mRNA was purified from total RNA using oligo-linked poly-T magnetic beads. Fragmentation was carried out using divalent cations at elevated temperature in NEB Next First Chain Synthesis Reaction Buffer (5x). The first strand of cDNA was synthesized using random hexameric primers and M-MuLV reverse transcriptase. The synthesis of the second strand cDNA was subsequently performed using DNA polymerase I and RNase H. The cDNA was size selected, adapter-ligated, and the products selectively enriched. Clustering of the index-coded samples was performed on a cBot clustering system using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, NEB) according to the manufacturer's instructions.

RNA-seq data analysis

After cluster generation, cDNA libraries were sequenced on Illumina HiSeq 1500 to obtain 2 × 150 bp pair-end reads at the INDEAR service facilities (Rosario, Argentina). Briefly, de novo transcriptome was assembled with all the reads, then the gene expression in each condition was calculated using the counts per million (CPM) of reads. RNA-Seq read quality was verified using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The readings of the three ontogenetic stages of *T. frezii* were performed and compared (basidiospores, hyphae, and teliospores).

Homology and identity of tubulins and tubulin-modifying enzymes of *T. frezii* against *U. maydis*, *A. flocculosa*, and *T. thlaspeos*

The mRNA sequences obtained from the RNA-Seq analysis were converted by searching open reading frames into hypothetical protein sequences using the ORF Finder site (https://www.ncbi.nlm.nih.gov/orffinder/) at the NCBI NIH. From these deduced sequences, between them tubulins and tubulin-modifying enzymes, a Blast-type comparison analysis was performed (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM = blastp&PAGE_TYPE = BlastSearch&LINK_LOC = blasthome) against the NCBI NIH database for fungi and PSI-BLAST (https://www.ebi.ac.uk/Tools/sss/psiblast/) against the UniProtKB/Swiss-Prot (the manually annotated section of UniProtKB), using default parameters. The focus was on the identification of α, β, and γ- tubulins as well as TTL, CCP1, and HDAC6 proteins (tubulin-modifying enzymes).

By aligning fungal tubulins and fungal tubulin-modifying enzymes belonging to fungus from the same class of *T. frezii* and using the basic local alignment search tool (BLASTP), we found conserved sequences that allowed us to identify the corresponding from *T. frezii* orthologues. Identity and similarity of tubulins and tubulin-modifying enzymes of *T. frezii* were compared with those of *U. maydis*, *Antracocystis flocculosa*, and *Thecaphora thlaspeos*. They were analyzed by multiple sequencing and protein pair alignments, omitting SP if present (Network protein sequence analysis: CLUSTALW n.d., Thompson et al. 1994). Proteins with ≥ 25% identity and an amino acid alignment length greater than 80 were considered homologous (Sander and Schneider 1991).

qPCR analysis of *T. frezii* tubulins and tubulin-modifying enzymes

The quantitative study of transcripts from genes corresponding to tubulins (α, β, and γ-tubulins), TTL, cytosolic carboxypeptidase 1 (CCP1), and histone deacetylase 6 (HDAC6) was carried out by real-time PCR. The oligonucleotide list for the genes was designed using the Primer-Blast program (NCBI, NIH; Table 1). Quantification of gene expression was performed using the StepOne Plus® real-time PCR detection system (ThermoFisher, MA, USA).

The cDNA was prepared from the same mRNA samples used for the RNA-Seq analysis, using the reverse transcriptase enzyme SuperScript® III (Invitrogen; Thermo Fisher Scientific™) and following the manufacturer's recommendations. The qPCR was performed with three technical replicas using the Sybr® Green Master Mix kit (Applied Biosystems; Thermo Fisher Scientific), following the manufacturer's recommendations.

Relative gene expression was performed using actin transcription as a reference gene for expression normalization [this gene is widely used for the normalization of fungal gene expression (Mosquera et al. 2009, Song et al. 2020)]. The program used for all the objectives was: 95°C for 3 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. Fluorescence was read after this step. At the end of the program, the temperature was reduced from 95 to 65°C in a speed ramp of 0.1°C/s, which allowed the evaluation of the melting curves for each reaction. The specificity of the amplicons was verified by analysis of the melting curves and sequencing of the fragments obtained. The change of expression in the target gene in relation to the expression of actin was calculated using the the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Then the means and SE (±) were determined for each of the different samples.

Table 1. Real time PCR oligonucleotides.

GenBank accession number	Gene name	Primer forward (5'→3')	Primer reverse (5'→3')	Amplification size (bp)
MW595346	α-tubulin	AGTTCGACCTGCTCTACTCG	CGACCTCCTCGTAGTCCTTC	126
MW595347	β-tubulin	CCGAGAACTGTGACATGCTG	GTGGCATTGTAGGGTTCGAC	187
MW595348	γ-tubulin	TTTCCCAACTCGGAGGAGAC	GGTTGGTTTGGCTGTAGCTC	175
MW595353	TTL	CGGTGCAAGAGGTCAAGAAG	GTTTGGACGAGCAGATCCAC	136
MW595352	CCP1	GACTTTGCGCTCGAATGGAT	ATCGAGCACCAAACTCATGC	171
MW595351	HDAC6	GTCGTTCAATCCGCACAAGA	CATCGTTGTCCATCGTCGAG	155
MW602863	Actin	CTACGTTGCCCTCGACTTTG	CGTTTCCGACAGTGATGACC	107

Statistical analysis

Statistical analysis was performed using InfoStat software (Di Rienzo et al. 2017). All data were calculated as mean ± standard deviation. Data were analyzed for statistical significance using the *t*-test.

Results

Detection of tubulins

Protein fractions of the three development stages of *T. frezii* (75 μg) were fixed on nitrocellulose membranes and incubated with specific antibodies for different tubulins.

First, we evaluate the presence of α-tubulin in the three developmental stages of *T. frezii* using a monoclonal antibody DM1A. This tubulin subtype was not detected in dot blot assays, neither in the cytosol nor in the membrane fractions. To elucidate why the DM1A antibody does not detect *T. frezii* α-tubulin, we compared the sequence recognized by the anti-α-tubulin antibody, DM1A clone with α-tubulin sequence from *T. frezii*. Figure 1 shows the epitope recognized by the DM1A antibody, where some differences can be observed with the α-tubulin of *T. frezii*.

The three stages of development: teliospores, basidiospores, and hyphae showed a positive reaction with the anti-Glu-Tub antibody, both in the cytosolic fraction and in membrane proteins (Fig. 2A). The only fraction that did not show the presence of Glu-Tub was the AP of the triton X-114 treatment in hyphae (Fig. 2A).

When samples were confronted with the monoclonal anti-Tyr-Tub antibody, it was observed that only proteins present in DP showed positive recognition. None of the remaining fractions, neither the cytosolic, nor AP, revealed the presence of Tyr-Tub. This result was repeated in teliospores, basidiospores, and hyphae (Fig. 2B).

To assess the hydrophobic nature of the tubulins that appeared in the membrane fraction, we evaluated the effect of alkaline carbonate treatment on tubulin present in the teliospore membrane. Thus, membrane pellet was first treated with carbonate pH 12, and then incubated with Triton X-114, to obtain AP and DP. Figure 2(C) shows that Glu-Tub appears only in the carbonate fraction (CP), not in the AP or DP of Triton X-114, demonstrating the hydrophilic character of this tubulin.

The presence of acetylated tubulin was assessed using the mouse monoclonal antibody 6–11B-1. Similar to DM1A for α-tubulin, acetylated tubulin was not detected in either the cytosol or the membrane, regardless of developmental stages of *T. frezii*. This result could be explained at least by two possibilities, (a) the absence of tubulin acetylase, the enzyme that load acetyl group in lysine-40 of α-tubulin; or (b) the lack of lysine in position 40, the specific site for acetylation. In order to investigate these theories, we use data from RNA-Seq analyzes of *T. frezii* tubulin. Here, we compared the amino acid sequence between position 1 and 55 of α-tubulins, brain, and *T. frezii*, with the aim to evaluate whether the lack of recognition of α-tubulin by the monoclonal antibody 6–11B-1 is related to the absence of the amino acid lysine at position 40 in the chain. The comparison of both sequences shows that α-tubulin of *T. frezii* did not contain the lysine-40 like neuronal tubulin (Fig. 3).

Next, we used immunofluorescence to visualize the presence of microtubule network in both, hyphae and basidiospores using specific antibodies for each isotype of tubulin. According with the results obtained in the dot blot assays, microtubules of Glu-Tub are present in hyphae and basidiospores (Fig. 4A and B). However, neither Tyr-Tub nor α-tubulin (clone DM1A) could be detected by immunofluorescence in either of the two incubated ontogenetic stages (Fig. 4C–F).

Homology, identity, and expression of tubulins and tubulin-modifying enzymes of *T. frezii*

Using the sequences obtained by RNA-Seq analysis, we design specific oligonucleotides to quantify the expression of the different tubulins and tubulin-modifying enzymes. A total of six transcripts encoding these proteins were identified. All the sequences obtained were deposited on the GenBank database (the accession numbers were in Table 1).

As seen in Fig. 5, α, β, and γ-tubulin are expressed in the three ontogenetic stages of *T. frezii*, although quantitatively different expressions of each one was found. The difference in the expression of each tubulin (α, β, and γ) in the different ontogenetic stages is statistically significant ($P < .02$), with the α-tubulin between basidiospores and teliospores.

The complete deduced sequence of three tubulins was obtained, with a total of 448 amino acids for α-tubulin, 447 amino acids for β-tubulin, and 454 amino acids for γ-tubulin (Table 2).

The comparative analysis with the complete sequence of tubulins from other fungi, revealed a high percentage of identity and homology with *A. flocculosa*, *U. maydis*, and *T. thlaspeos* (Table 2). The α-tubulin has 95% identity and 97% homology with the protein of the fungus *A. flocculosa*, 93% and 96% with α-tubulin from *U. maydis*, and 95% and 96% with *T. thlaspeos*, respectively. For β-tubulin, 92% identity with *A. flocculosa* and *U. maydis*, and 91% compared with *T. thlaspeos*, while the homology was the same for the three fungi (96%). The γ-tubulin presented high identity (94%–98%) and homology (97%–98%) with the three fungi.

The level of expression of three enzymes that could be involved in PTMs of tubulins was measured in the three biological stages of *T. frezii* by RT-PCR. Figure 6 shows the expression level of these enzymes TTL, CCP1, and HDAC6, where the highest expression was observed in the basidiospore stage. For each of these enzymes, the

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α-Tubulin of brain:          426  AALEKDYEEVGVDSVEGEGEEEGEE     450
α-Tubulin of T. frezii:      424  AALEKDYEEVGLDSVETEEEVLDY       448
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Figure 1. Comparison of the sequence that recognizes the anti-α-tubulin clone DM1A antibody with the sequence of α-tubulin from *T. frezii*. The amino acid in dark red indicates the amino acids that do not match in *T. frezii*.

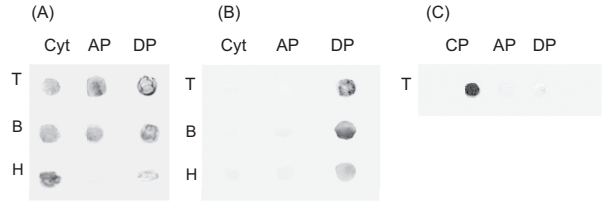

Figure 2. Reactivity of anti-isotypes of α-tubulin antibody against different ontogenetic stage of *T. frezii*. Protein fractions of teliospores (T), basidiospores (B), and hyphae (H) were applied directly onto nitrocellulose membrane and incubated with antispecific α-tubulin primary antibody. (A) Reactivity of antidetyrosinated tubulin (Glu-Tub), (B) reactivity of antityrosinated tubulin (Tyr-Tub), and (C) reactivity of antidetyrosinated tubulin (Glu-Tub) against teliospore membrane proteins previously treated with carbonate. Cyt: cytosol proteins, AP: aqueous phase of Triton X-114; DP detergent phase of Triton X-114, and CP: carbonate-extracted protein.

Table 2. Sequence analyzes of *T. frezii* tubulins: length, homology, and identities were compared to *U. maydis*, *A. flocculosa*, and *T. thlaspeos* orthologues.

Tubulin genes	Deduced protein length (number of amino acids)	Number of amino acids—percentage of identity (%)/percentage of homology (%)		
	T. frezii	*U. maydis*	*A. flocculosa*	*T. thlaspeos*
α-TUB	448	448- 93/96	448- 95/97	448- 95/96
β-TUB	447	448- 92/96	448- 92/96	448- 91/96
γ-TUB	454	454- 94/97	454- 98/98	454- 97/98

Table 3. Sequence analyzes of *T. frezii* tubulin-modifying enzyme genes: TTL, CCP1, and HDAC6. Length, homology, and identities were compared to *U. maydis*, *A. flocculosa*, and *T. thlaspeos* orthologues.

Enzyme genes	Number of amino acids deduced	Number of amino acids- percentage of identity (%)/percentage of homology (%)		
	T. frezii	*U. maydis*	*A. flocculosa*	*T. thlaspeos*
TTL	519	513–55/68	543–59/68	514–61/73
CCP1	401	432–72/81	424–79/84	411–79/80
HDAC6	799 (partial)	1461–70/80	1482–84/89	1538–80/85

expression differences in each stage are significant with $P < .02$; except for TTL between the teliospore and hyphae stages, where there is no significant difference.

Table 3 shows a summary of the RNA analysis of these enzymes and comparison with their corresponding orthologues. Homology and identity of the *T. frezii* enzymes with the fungi *A. flocculosa*, *U. maydis*, and *T. thlaspeos* were found in all the analyzed enzyme sequences. In the case of TTL, a sequence of 519 amino acids could be identified, with moderate values of identity and homology with three fungi (55%–61% and 68%–73%, respectively). CCP1 an enzyme of 401 amino acids had a moderate percentage of identity and homology (72%–79% and 80%–84%). In addition, a transcript for HDAC6 was found, with identity and homology percentages between 70%–84% and 80%–89%, respectively.

Discussions and conclusions

Thecaphora frezii is the causative agent of peanut smut, a disease that produces great economic losses in the agriculture of Argentina. Little has been described about the biology of this fungus, it is known that it produces teliospores, resistance structures that remain in the soil and germinate producing probasidium, basidium, basidiospores, and infective hyphae by conjugation of compatible hyphae (Rago et al. 2017).

In this work, we focus on the study of α-tubulin to identify the existence of PTMs and its cellular location. We evaluated the presence of α-tubulins in cytosol and membrane protein fractions of the three structures of *T. frezii* (teliospores, basidiospores, and hyphae) using dot blot with specific antibodies, immunostaining, and correlated the results with data obtained from RNA-Seq analysis.

When protein extracts of *T. frezii* were incubated with the DM1A antibody, no specific recognition of tubulin was observed. Considering that this antibody recognizes most of the α-tubulins present in eukaryotic cells, the lack of reactivity of DM1A against any of the protein fractions of the three developmental stages of *T. frezii* was an unexpected result. This antibody recognizes the amino acid sequence 426–451 of the carboxy terminal end of α-tubulin, with fragment 426–430 being particularly important (Breitling and Little 1986). Although certain degree of homology with the deduced sequence of *T. frezii* was observed, it would be not enough to be recognized by the antibody. The difference in the sequence between both α-tubulins could be one of the reasons for the lack of recognition of DM1A. Another reason could be the denaturation of tubulin during the extraction process. This last explanation is reinforced by the work of Breitling and Little (1986), who described that DM1A is more reactive with the native tubulin dimer than with the denatured α subunit, and that it has low reactivity with peptides. Furthermore, Breitling and Little (1986) suggest that the anti-DM1A antibody has a discontinuous or conformational epitope and that C-terminal tyrosination has no effect on antigenicity. This could also explain the absence of fluorescence in immunostaining of hyphae and basidiospores. It is probable that the processing of the samples results in the loss of folding of the protein, modifying the site of the epitope that the antibody recognizes.

The C-terminus of α-tubulin is characterized by two glutamic residues followed by an aromatic amino acid (tyrosine or phenylalanine) in eukaryotic cells. The complete deduced sequence of α-tubulin of *T. frezii*, 448 amino acids, confirmed that the last three amino acids are leucine, aspartate, and tyrosine; this could be demonstrated in the dot blot assay. Using the TUB1A2 antibody, that recognizes the tyrosine residue at the carboxyl terminal end

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T.frezii    1 MREVLSLHVGQAGIQIGNACWELYLAEHGLSPDGRILEGSP-SQHDDGFSTFFSET   55
Identity      MRE :S:HVGQAG:QIGNACWELY  EHGL:.DG:: ...  .  DD.F.TFFSET
Human       1 MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSET   56
```

Figure 3. Sequence of aminoacids 1–55 of α-tubulin in brain and α-tubulin of *T. frezii*. The amino acid in dark red indicates the position 40 in the chain.

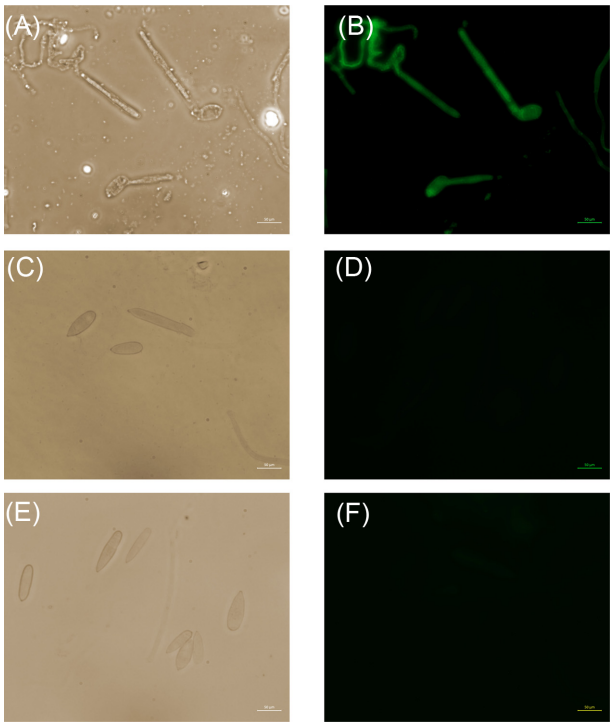

Figure 4. Immunodetection of *T. frezii* α-tubulin. Fresh hyphae and basidiospores were incubated with anti-α-tubulin antibodies. (A) and (B): antidetyrosinated tubulin antibody, (C) and (D): antityrosinated tubulin antibody, and (E) and (F): anti-α-tubulin clone DM1A antibody. (A), (C), and (E): bright field microscopy and (B), (D), and (F): fluorescence microscopy. Magnification 60x.

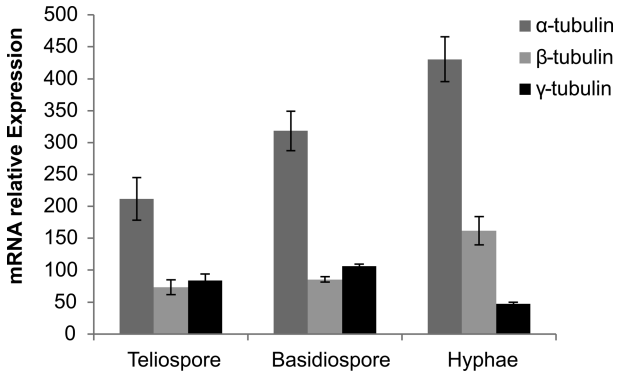

Figure 5. *Thecaphora frezii* tubulins relative mRNA expression levels according to its ontogenetic stage (teliospore, basidiospore, and hyphae). RNA was isolated and used to measure gene expression by RT-PCR. Data represent average values from technical triplicates ± standard deviation and are expressed relative to values of actin gene transcription.

of α-tubulin, we only detected this tubulin as membrane protein but not in cytosolic fraction in all stages of fungal development.

Wolff (2009) described that a part of the tubulins extracted from some membranes are hydrophobic, which remains in the detergent phase of Triton X-114, while another part remains in

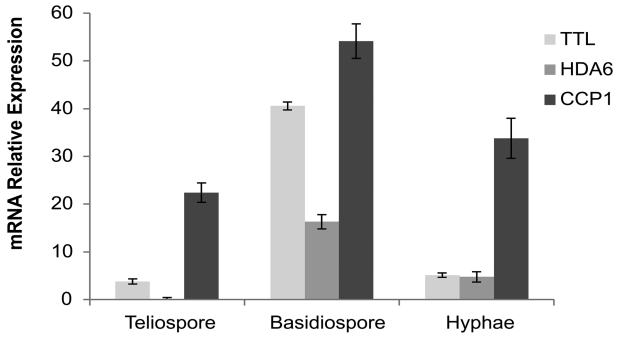

Figure 6. *Thecaphora frezii* relative mRNA expression levels of tubulin-modifying enzymes according to its ontogenetic stage (teliospore, basidiospore, and hyphae). TTL, CCP1, and HDAC6. RNA was isolated and used to measure gene expression by RT-PCR. Data represent average values from triplicates ± standard deviation and are expressed relative to values of actin gene transcription.

the cytoplasmic AP, citing as an example liver and kidney membranes that contain a small amount of hydrophobic tubulins. We detected Tyr-Tub in DP, possibly with hydrophobic characteristics, according to Wolff (2009). Although the presence of cytoskeleton structures and tubulins in membranes has been described (Por et al. 1991), this result was unexpected, since Tyr-Tub was usually associated to cytosolic tubulin or at highly dynamic microtubules (Andrieux et al. 2019). Zekert and Fischer (2009) detected tyrosinated tubulin in cytoplasm and mitotic spindles of *A. nidulans*. On the other hand, microtubules containing Tyr-Tub were not detected in *T. frezii* by immunofluorescence technique. This result is probably due to the fact that Tyr-Tub is associated to membrane protein as simple protein and not as microtubule form, therefore could not be detected by the antibody.

Most organisms synthesize Tyr-Tub, which can be post-translationally modified. The presence of genes encoding tyrosine tubulin in *T. frezii* was verified (data not shown) by RNA seq. Tubulin tyrosine carboxypeptidase can remove this amino acid producing detyrosinated tubulin or Glu-Tub. This phenomenon is reversible since, TTL, a well-characterized enzyme, catalyzes retyrosination (Westermann and Weber 2003, Zekert and Fischer 2009, Janke 2014). The presence of this PTM in *T. frezii* was detected in the three stages of development using the dot blot test. In basidiospores and teliospores, Glu-Tub was detected in both phases AP and DP. However, in hyphae, the infective stages of fungus, was only detected in DP. The presence of Glu-Tub associated to membrane in hyphae, could suggest a specific role of this tubulin in the infective process. In the immunofluorescence images, we could observe that the entire cytoplasm of the hyphae and basidiospores were decorated with Glu-Tub.

PTMs such as detyrosination, acetylation, and polyglutamylation were shown to occur preferentially in microtubules whose population can have various modifications and combinations of PTMs. Detyrosination and acetylation are associated with the longevity of microtubules, although it does not imply that they have greater stability (Janke 2014, Song and Brady 2015, Kesarwani et al. 2020). Whether detyrosination of microtubules makes them more flexible or alters the interaction with associated proteins is

unknown, as Glu-Tub is associated with flexing in muscle contraction (Janke and Magiera 2020). Formulating hypotheses about the role that these long-lived microtubules play in the infective process of *T. frezii* or their biological function is uncertain, since no differences were observed between the three ontogenic stages of the fungus.

Zekert and Fischer (2009) showed for the first time the existence of detyrosinated microtubules in *A. nidulans*. They observed that tyrosinated and detyrosinated microtubules exist in parallel in interphase cells and tyrosinated microtubules depolymerizes, while detyrosinated microtubules remains in mitotic cells. In addition, they suggest that these microtubules are independent of the growth phase, which is consistent with our results, since we did not find differences in the tubulins of the three stages of *T frezii*. Detyrosination can affect indirectly microtubule growth speed and persistence, also regulates the active disassembly of microtubules by regulating the MAPs that affect microtubule stability (Janke and Magiera 2020). It is well-known that the motor protein Kinesin-1 binds preferentially to detyrosinated microtubules, for more efficient transportation as well are involved in nuclear oscillations in *Saccharomyces cerevisiae* (Hammond et al. 2008, Zekert and Fischer 2009). Zekert and Fischer (2009) shown that motor protein UncA of the kinesin-3 family transports vesicles and endosomes along a subpopulation of detyrosinated microtubules in contrast to other kinesins, and these vesicles are required for fast hyphae extension. These authors assume that these stable microtubules might be important for hyphal extension during mitosis. Although there are no reports on the growth of *T. frezii*, we suppose that this detyrosination could be fundamental in the transport of vesicles to maintain the development of basidiospores and hyphae. On the other hand, in teliospores, tubulins would be involved in maintaining the organization of the cytoplasm, nucleus, and organelles, since they are not dividing cells.

Acetylated tubulin was not detected in either the cytosol or the membrane fraction of any of the three structures of *T. frezii*. The lack of reactivity to the 6–11B-1 antibody was due to changes in the primary sequence of the molecule. With the deduced sequence of α-tubulin obtained by the RNA-Seq analysis, we could observe that there is a serine instead of lysine in position 40, which corroborated the negative results obtained in the dot blot assay. These results coincide with those described by Zekert and Fischer (2009), who did not detect acetyl tubulin in the filamentous fungi *A. nidulans*. Westermann and Weber (2003) also describe that there are no modifications such as acetylation, polyglutamination, and polyglycylation in *S. cerevisiae* or filamentous fungi.

The term membrane tubulin includes integral tubulin and peripheral tubulin as free dimers, in complexes, or as microtubules attached to membranes directly or via proteins or lipid modifications but requiring detergent extraction. Beltramo et al. (1994) showed that brain membranes contain tubulins that behave as integral membrane proteins, since they appear localized in the phase rich in Triton X-114. This hydrophobic membrane tubulin is enriched in the acetylated subtype. However, whether these membranes were first treated with alkaline solution, a condition to remove only associated membrane proteins, the tubulin appear in aqueous rich phase like hydrophilic cytosolic tubulin. This demonstrates that the hydrophobic behavior was due to an interaction with an integral membrane protein such us Na-K-ATPase (Alonso et al. 1998). We detected the presence of Tyr-Tub and Glu-Tub in the detergent-rich phase, suggesting a behavior similar to that of an integral membrane protein. However, after treating teliospore membranes with sodium carbonate at pH: 12, we observed that Glu-Tub disappear from DP and shows hydrophilic behavior like cytosolic tubulin, as described by Beltramo et al. (1994). This result allowed us to establish that, although Glu-Tub behaves as an integral membrane protein in teliospores, this behavior is probably due to the association with some membrane component.

Most of the peripheral membrane tubulins are bound on the cytoplasmic side; this interaction could be through integral tubulins receptors or receptor-bound proteins or lipid rafts (Wolff 2009). Proteins such as HPC-1/Syntaxin A, a neuronal membrane protein, which binds to tubulin dimers but not to microtubules, have been described. This protein produces shortening and assembly inhibition, regulating the release of neurotransmitters. CLIP-59 (cytoplasmic binding protein) also binds tubulin dimers preferentially to microtubules and appears to be involved in microtubule anchoring (Wolff 2009). Alonso et al. (1998) identified an enzyme Na+, K+-ATPase that interacts with acetylated tubulin giving the hydrophobic characteristic, which causes it to migrate to the membrane in neurons. The association between plasma membrane Na+, K+-ATPase, and acetylated α-tubulin occurs in various cell lines and tissues. Another hydrophobic complex between acetylated tubulin and H+-ATPase has been found in *S. cerevisiae*, and this membrane tubulin inhibits the enzyme. (Wolff 2009). The activities of both these ATPases are restored upon dissociation of the acetylated tubulin/ATPase complex. Monesterolo et al. (2008) reported a plasma membrane Ca2+-ATPase in rat brain vesicles, which forms complexes with acetylated tubulin and regulates enzyme activity by association/dissociation with acetylated tubulin. They also showed that this regulation is dependent on membrane lipid composition and tubulin concentration (Monesterolo et al. 2012). The composition of proteins that are part of the membrane of *T. frezii* is not known, so it is unknown if it has ATPases or receptors through, which tubulins can interact with the membrane.

Having shown that tubulin binds to the membrane through a hydrophobic interaction, that it is not an integral part of the membrane, and that we only find tyrosinated and detyrosinated tubulin in the membrane, one could speculate about the existence of some ATPase-type enzyme, or some other membrane component that interacts with these tubulins. Studies to demonstrate these are in progress. On the other hand, Wolff (2009) summarizes the work of different authors where they report that tubulin interacts with lipid vesicles; this interaction depends on the polar head of the phospholipid. Neutral lipids and positively charged lipids favor the interaction, while negatively charged phospholipids interfere with the interaction. This interaction can be directly with tubulin or through MAPs. Additional studies are required to evaluate this interaction of tubulins with membrane components, for which it is necessary to advance in the study of membrane proteins and lipids of *T. frezii*.

Some possible functions of membrane tubulins or microtubules have been discussed by Wolff (2009), such as regulation of membrane fluidity or microviscosity, regulation of Na+K+ATPase and other membrane ATPases by tubulin acetylated. Tubulins can interact with phospholipases linked to receptors or act as a GTP exchanger in certain receptors, intervening in the regulation of functions. It is still unknown if the regulations of the different functions are specific for the different tubulin isotypes and their PTMs.

Considering RNA-Seq data, which shows the presence of Tyr-Tub gene but not the gene encoding Glu-Tub, the identification by specific immunochemical recognition of Tyr-Tub and Glu-Tub in *T. frezii* suggests the existence of PTMs of tubulin. Based on these results, we analyzed the presence of genes that synthesize the enzymes involved in these modifications in *T. frezii*. We found the sequence of TTL expressed mainly in basidiospores, and less in

teliospores and hyphae. We also found CCP1, an enzyme that remove a glutamic residue from the carboxyl terminal end of detyrosinated microtubules, irreversibly, forming delta 2 tubulin; this prevents other PTMs such as tyrosination and polyglutamylation (Song and Brady 2015). Rogowski et al. (2010) demonstrate that CCP1 is not involved in α-tubulin detyrosination; it removes the C-terminal glutamate residue from detyrosinated α-tubulin, and the glutamate side chains of α and β-tubulin (Berezniuk et al. 2012). Tanco et al. (2014) showed that CCP1 processes glutamates and aspartate at C-terminal residues. The deduced sequence carboxyl terminal end of α-tubulin *T. frezii* shows a tyrosine followed by aspartate; CCP1 could thus cut this aspartate residue generating delta 2 tubulin.

We were unable to determine if there is another carboxypeptidase that cleaves the tyrosine residue of tubulin to give rise to detyrosinated tubulin, but we detected a carboxypeptidase that acts on glutamic residues. It could be that there is also delta 2 and delta 3 tubulin. With these results, we wonder if the polyclonal antibody used in this study could recognize other modified tubulins, since *T. frezii* α-tubulin sequence does not completely match the epitope of the antibody and that the intensity of development was higher for Glu-Tub.

TTL is not able to tyrosinate microtubules, since the enzyme depends on a specific conformation of the α/β-tubulin dimer, must first be disassembled for retyrosination of their dimers before their reassembly, while carboxypeptidase has a preferential action on microtubules, which is demonstrated by the existence of microtubules with older domains rich in detyrosinated tubulins and ends rich in tyrosinated tubulins (Kesarwani et al. 2020, Song and Brady 2015). We speculate that Glu-Tub detected in the cytosol is found as part of the microtubules and not as free tubulin; therefore, it could not be retyrosinated by TTL. This could be the reason why we did not detect cytosolic Tyr-Tub of any of the three developmental structures in the dot blot assay or by immunofluorescence. The tyrosination and detyrosination cycle depends on equilibrium between the two modifying enzymes (Zekert and Fischer 2009); it could be that there is more detyrosination because the carboxypeptidase showed a higher level of expression than TTL in the three ontogenic stages studied.

The data obtained through RNA-Seq analysis allowed us not only to deduce the sequence of α, β, and γ-tubulin from *T. frezii*, but also analyze the variation level of expression according to fungus stage. In hyphae, the expression of α-tubulin was approximately 50% more than in basidiospores and twice that expressed in teliospores. With respect to β-tubulin, the amount expressed in hyphae is approximately double that found in basidiospores and teliospores, while the expression of γ-tubulin is the majority in basidiospores, being double that in hyphae. It is necessary to take into account that mRNA levels may not be directly related to protein levels due to numerous possible PTMs.

From the respective deduced protein sequences, we evaluate the homology and identity with those from other related species (from the phylogenetic and pathogenicity point of view: *U. maydis*, *A. flocculosa*, and *T. thlaspeos*). Comparative analysis shows a high degree of identity with tubulins from *A. floculosa* (between 92% and 98%), *U. maydis* (92%–94%), and with *T. thlaspeos* (91%–97%).

When enzymes involved in PTMs of tubulin were analyzed, we could find three related enzymes. These enzymes also showed moderately to high identity and homology percentages when compared with the phytopathogenic fungi *U. maydis* and *T. thlaspeos* and with the nonpathogenic fungus *A. flocculosa*. In these fungi, there are no known studies of PTMs of tubulins.

The intracellular location of tubulins with PTMs could help to understand the possible functions of these modified tubulins. Further experiments are required to understand the biological importance of microtubule location and modifications. Why these forms of Glu-Tub and Tyr-Tub are bound to membrane remains to be elucidated. The characterization of this pathogen will allow having more tools for the design of different control strategies based on the biology of the fungus, e.g. blocking the enzymes that modify tubulins or the binding of tubulin to the membrane could alter specific functions of specific enzymes or receptors, thus blocking the normal development of the fungus and therefore the infection.

This is the first study to identify the tubulins present in *T. frezii*, their location and the expression of the mRNAs that codes for these tubulins and specific enzymes that modify them. We demonstrate the presence of detyrosinated and tyrosinated tubulin in the membrane of the three ontogenic stages of *T. frezii*. This membrane tubulin is hydrophilic in nature but form a complex with some component of the membrane, acquiring a hydrophobic behavior. The ligand that allows it to associate with the membrane is not known and the function of this membrane tubulin is also unknown. In the cytosol, we only detected the presence of detyrosinated tubulin, regardless of the stage of development, indicating the presence of longer-lived microtubules. Through RNA seq studies we were able to deduce the sequence of α, β, and γ-tubulins, which had high homology to other fungi belonging to the same genus, in addition to their expression level as well as the sequence of enzymes TTL, CCP1, and HADC 6, that are involved in these PTMs that we detected.

Conflict of interest statement. None declared.

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