Universidade do Minho
Escola de Medicina

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Sporothrix brasiliensis genetic toolbox

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Doutor Fernando José Santos Rodrigues

e de

Doutora Cristina Amorim Cunha

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Sporothrix brasiliensis genetic toolbox

Abstract

Sporotrichosis is the world's most prevalent and distributed subcutaneous mycosis. *S. brasiliensis* species is reported as the most virulent species, exhibiting the worst clinical manifestations, but the infection and virulence mechanisms of *Sporothrix* spp. still lack clarification. During the last years, the genome of several species has been fully sequenced, and the data is available in open access resources. However, this genomic knowledge has not been accompanied by reverse genetics tools, which could contribute to decoding gene functions. This present work focuses on creating a genetic toolbox, allowing the development of a set of transformants that can ultimately be used in future studies to unravel the poorly understood virulence mechanisms of *S. brasiliensis*. For that, we tested a CRIPR/Cas9 system for *S. brasiliensis*, however our results suggest that the TEF1-α promoter, present in the plasmid used, pPTS608, engineered for *Aspergillus* spp., does not express the CRISPR/Cas9 machinery in the *S. brasiliensis* biological system. Therefore, our work moved to evaluating the usefulness of different promoters and develop a set of *S. brasiliensis* transformants. Our results show that the expression promoted by the GAPDH promoter led to a high genetic and constitutive expression of fluorescence proteins in *S. brasiliensis* transformants. Thus, we developed a set of plasmids for cloning with the GAPDH or H2A promoter driving the expression of fluorescent proteins (GFP and mCherry). These plasmids allow the cloning of any gene for expression in an N-terminal in-frame fusion with red or green fluorescent proteins. Accordingly, *S. brasiliensis* transformants show bright fluorescence localized in the nucleus when an in-frame fusion was made with the endogenous histone H2A, indicating the suitability of these plasmids for gene cloning and in-fusion fluorescent proteins. Together, our results confirm the success in constructing easy-to-edit plasmid protocol's and the creation of a set of *S. brasiliensis* transformants through the *Agrobacterium tumefaciens*-Mediated Transformation (ATMT). This genetic toolbox can be used in the future in order to elucidate the poorly understood virulence mechanisms of *S. brasiliensis*.

Key-words: *Sporothrix brasiliensis*, CRISPR/Cas9, fluorescent-proteins, plasmid.

Sporothrix brasiliensis caixa de ferramentas genéticas

Resumo

A esporotricose é a micose subcutânea mais prevalente e distribuída no mundo. A espécie *S. brasiliensis* é descrita como sendo a mais virulenta, estando associada com manifestações clínicas mais severas, contudo, os mecanismos subjacentes à infeção e virulência de *Sporothrix* spp. ainda carecem de clarificação. Durante os últimos anos, o genoma de várias espécies de *Sporothrix* foi totalmente sequenciado e os dados estão disponíveis em recursos de acesso aberto. Em contraste, esse conhecimento genómico não foi acompanhado pelo desenvolvimento de ferramentas de genética reversa, que poderiam contribuir para a decodificação das funções dos genes. O presente trabalho focou-se na criação de uma caixa de ferramentas genéticas, permitindo a criação de uma biblioteca de transformantes que podem ser usados no futuro em estudos com o objetivo de desvendar os mecanismos pouco conhecidos de virulência do *S. brasiliensis*. Para tal, testamos um sistema CRIPR/Cas9 para *S. brasiliensis*. Contudo, os nossos resultados sugerem que o promotor TEF1-α, presente no plasmídeo utilizado, pPTS608, desenhado para *Aspergillus* spp., não é capaz de expressar a maquinaria CRISPR/Cas9 no sistema biológico de *S. brasiliensis*. Neste sentido, o nosso trabalho focou-se na avaliação do potencial de diferentes promotores e no desenvolvimento de um conjunto de transformantes de *S. brasiliensis*. Os nossos resultados mostram que a expressão induzida pelo promotor GAPDH resultou numa alta e constitutiva expressão genética de proteínas de fluorescência em transformantes de *S. brasiliensis*. Assim, desenvolvemos um conjunto de plasmídeos para clonagem com o promotor GAPDH ou H2A para a expressão de proteínas fluorescentes (GFP e mCherry). Estes plasmídeos permitem a clonagem de qualquer gene para expressão em fusão N-terminal *in-frame* com proteínas fluorescentes vermelhas ou verdes. Os transformantes de *S. brasiliensis* apresentam fluorescência localizada no núcleo quando a fusão *in-frame* foi feita com a histona endógena H2A, demonstrando a utilidade desses plasmídeos para clonagem de genes e proteínas fluorescentes em fusão. Em soma, os nossos resultados confirmam o sucesso na elaboração de protocolos para a construção de plasmídeos fáceis de editar e na criação de um conjunto de transformantes de *S. brasiliensis* através da Transformação Mediada por *Agrobacterium tumefaciens* (ATMT). A criação desta caixa de ferramentas genéticas, irá permitir no futuro elucidar os mecanismos de virulência pouco conhecidos do *S. brasiliensis.*

Palavras-chave: *Sporothrix brasiliensis*, CRISPR/Cas9, proteínas-fluorescentes, plasmídeos.

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List of abbreviations

% – Percentage

°C – Degree Celsius

µg – Microgram

µL – Microliter

AIDS – acquired immunodeficiency syndrome

AS – Acetosyringone

ATMT – Agrobacterium tumefaciens-mediated transformation

BMDM – Bone marrow-derived macrophage

bp – Base pairs

cDMEM – Complete Dulbecco's modified Eagle's medium

CRISPR – clustered regularly interspaced short palindromic repeats

DAMP – Damage-associated molecular pattern

EDTA – Ethylenediamine tetra acetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

FACS – Fluorescence-activated cell sorting

FITC – Fluorescein isothiocyanate

gDNA – Genomic DNA

HygB – Hygromycin B

KO – Knockout

L.B. – Miller's L.B. Broth

LC-MS – Liquid Chromatography-Mass Spectrometry

MFI – Mean fluorescence intensity

mg – Milligram

ml - Milliliter

mL – Milliliter

MLS – Mitochondrial localization signals

MOI – multiplicity of Infection

mRNA - messenger RNA

ng – Nanogram

NLS – Nuclear localization signals

nt – Nucleotides

PAMP – Pathogen-associated molecular pattern

PBS – Phosphate buffered saline

PCR - Polymerase chain reaction

PCR – Polymerase chain reaction

R.T. – Room temperature

RNA - Ribonucleic acid

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

rpm - Rotations per minute

RT - Room temperature

SOC – Super optimal broth with catabolite repression

TLR – Tool-like receptor

WT – Wild type

YPD – Yeast extract peptone dextrose medium

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CHAPTER 1 – Introduction

Sporotrichosis is an acute or chronic granulomatous mycosis with a wide geographic distribution and the most prevalent subcutaneous mycosis worldwide (1,2). This disease has become a public health concern that will likely worsen in the future. It is currently a hyperendemic disease in countries like China, Mexico, Brazil, Peru, Venezuela, Colombia, and South Africa (1,3–5). The disease incidence and etiological agent vary according to geographic region, primarily based on case report observations (2). The sporotrichosis etiologic agents are fungi present in the pathogenic clade of the *Sporothrix* genus (1,6), where *S. brasiliensis* species is reported as the most virulent, exhibiting the worst clinical manifestations (7,8).

Until the mid-1990s, feline sporotrichosis in Brazil appeared only as sporadic, self-limiting clusters. However, over the last decades, the sporotrichosis epidemiological scenario changed drastically in Brazil, becoming the most endemic country in the world (1,3). Nowadays, feline sporotrichosis caused by *S. brasiliensis* has led to a large epidemic with zoonotic transmission in Brazil, becoming the principal etiological agent of feline and human sporotrichosis in the region (1,8–10). Cats play an essential role in the *S. brasiliensis* infection. They are highly susceptible to sporotrichosis and can easily transmit their disease to felines and humans through bites and scratches (3,11).

Despite the increasing incidence of sporotrichosis, it is unclear which virulence traits are involved in this disease's establishment, development, and severity. Therefore, the development of genetic engineering techniques for *S. brasiliensis* transformation, such as CRISPR/Cas9 and ATMT, are urgently needed to create a mutant's library to understand the association of specific genes and functions and unravel this microorganism pathophysiology.

Sporothrix **genus and sporotrichosis**

Sporotrichosis' first report was in 1898 by the medical student Benjamin Schenck (12), and today is the world's most prevalent and dispersed subcutaneous mycosis (5). This disease is endemic and caused by fungus from the *Sporothrix* genus, which is prevalent worldwide in tropical and subtropical areas (9), affecting immunocompetent (13) and immunodeficient hosts (14). The sporotrichosis etiological agents belong to the *Sporothrix* genus inside the Ophiostomatales order, generally associated with non-pathogenic environmental fungi strongly related to decaying wood, plants, and soil (15). However, members of this genus are successful thermally dimorphic mammalian pathogens and the causative agents of human and animal sporotrichosis (1,3,10,15). The mechanisms responsible for this are still poorly described (8).

This infection usually occur from transcutaneous trauma through the entry of fungal conidia into the host, either through contaminated plant material or through bites or scratches from cats with sporotrichosis (16). Once in the inoculation site, the fungi promote a skin lesion and an ulcerated nodule. The infection eventually spreads to other host organs, through the lymphatic system, causing the disseminated version of the disease with severe complications (2,17). The infections may progress into chronic cutaneous, subcutaneous, or even deeper conditions involving the lymphatics, fascia, muscles, cartilage, and bones (2).

Since its discovery over a century ago, sporotrichosis has been exclusively attributed to a single species, *Sporothrix schenckii*. However, the development of advanced phylogenetic molecular techniques in the last decade highlighted the role of other species as causative agents of sporotrichosis (7)**.** Further studies showed significant differences in morphological, physiological, genetic, epidemiological, virulence traits, and antifungal susceptibility among *Sporothrix* species. (7,18–24). These discoveries resulted in a division of the *Sporothrix* genus into two clades. Species inside the *Sporothrix* environmental clade are generally associated with an ecological transmission route and the sapronoses form of sporotrichosis (1,8). Inside the pathogenic clade, species are clinically relevant successful mammal pathogens, such as *Sporothrix schenckii, Sporothrix brasiliensis, Sporothrix globosa*, and *Sporothrix luriei* (15,16,25).

The etiological agents of sporotrichosis are categorized as ascomycetous thermally dimorphic fungi. They are abundant in the environment on substrates such as living and decaying vegetation, animal droppings, and soil (9,26). They also require a temperature of 22-27 °C, 90% humidity, soil rich in cellulose, and a pH between 3.5 and 9.4 to grow as sessile dematiaceous conidia together with hyaline sympodial conidia in their filamentous form (Figure 1A) (9,26). After traumatic inoculation, the fungi transit to the parasitic yeast form, growing as cigar-shaped yeast-like cells at 37 °C while infecting mammals (Figure 1B) (2,8). The success of *Sporothrix* infection in mammals lies in its ability to change from a saprophytic mycelial lifestyle in the environment to a parasitic yeast cell at an elevated temperature (15). Accordingly, how efficient this morphological transition occurs, and consequently the fungi pathogenicity, varies for each *Sporothrix* species and strain (8,19,25).

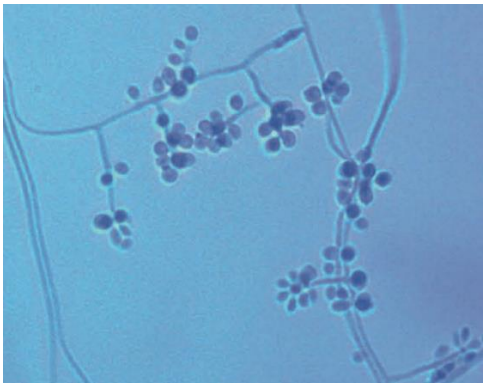
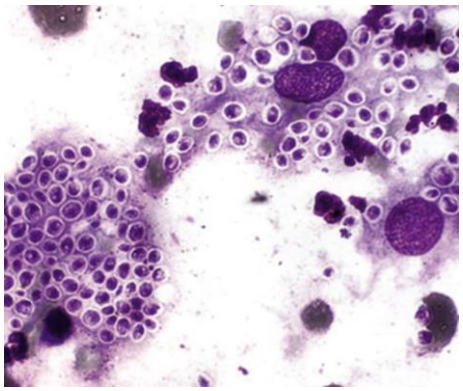
A  B 

Figure 1. *Sporothrix* spp. thermally dimorphic in microscopy. (A) Saprophytic morphology. Image shows a hyaline septate hypha, conidiophore that originates hyaline conidia in a bouquet arrangement. The sample was stained with cotton blue and magnified x400 in microscopy. Image from Orofino-Costa et al., 2017. (B) Smear from an ulcerated cat lesion, evidencing the presence of the *Sporothrix* spp. in its yeast-like form inside host cells. The smear was stained with panoptic staining and magnified 100x in microscopy—image from Sykes & Greene, 2011.

Several mammals are prone to sporotrichosis, such as humans, dogs, cats, mice, rats, chimpanzees, fishes, dolphins, and parrots (4,9). Although human sporotrichosis has a broad range of clinical manifestations, it can be classified into four types: fixed cutaneous, lymphocutaneous, disseminated cutaneous, and extracutaneous sporotrichosis (27). Itraconazole is the most used and recommended antifungal in treating sporotrichosis. However, resistance cases have already been reported (28) and are now considered an emerging health problem (29).

Sporotrichosis epidemiology

Sporotrichosis is not a mandatory notifiable disease in most regions, and its incidence is primarily based on case report documentation, while the actual prevalence is unknown (2,9). Nevertheless, this disease has been reported in the United States, South America (Brazil, Colombia, Guatemala, Mexico, Peru), Asia (China, India, Japan), and Australia (2,9) is currently a hyperendemic disease in regions of countries like China, Mexico, Brazil, Peru, Venezuela, Colombia, and South Africa (1,3–5).

The distribution of *Sporothrix* species is worldwide (Figure 2), especially in tropical and temperate climates (10). In most areas, more than 80% of cases are caused by a single predominant species, for example, Asia: *S. globosa* (99.3%); Australia and South Africa: *S. schenckii* (94%); south-eastern South America: *S. brasiliensis* (88%); and western South and Central America and North America: *S. schenckii*

(89%) (15). Except for a unique outbreak at the beginning of the nineteenth century in France, Sporotrichosis is less prevalent in Europe (3).

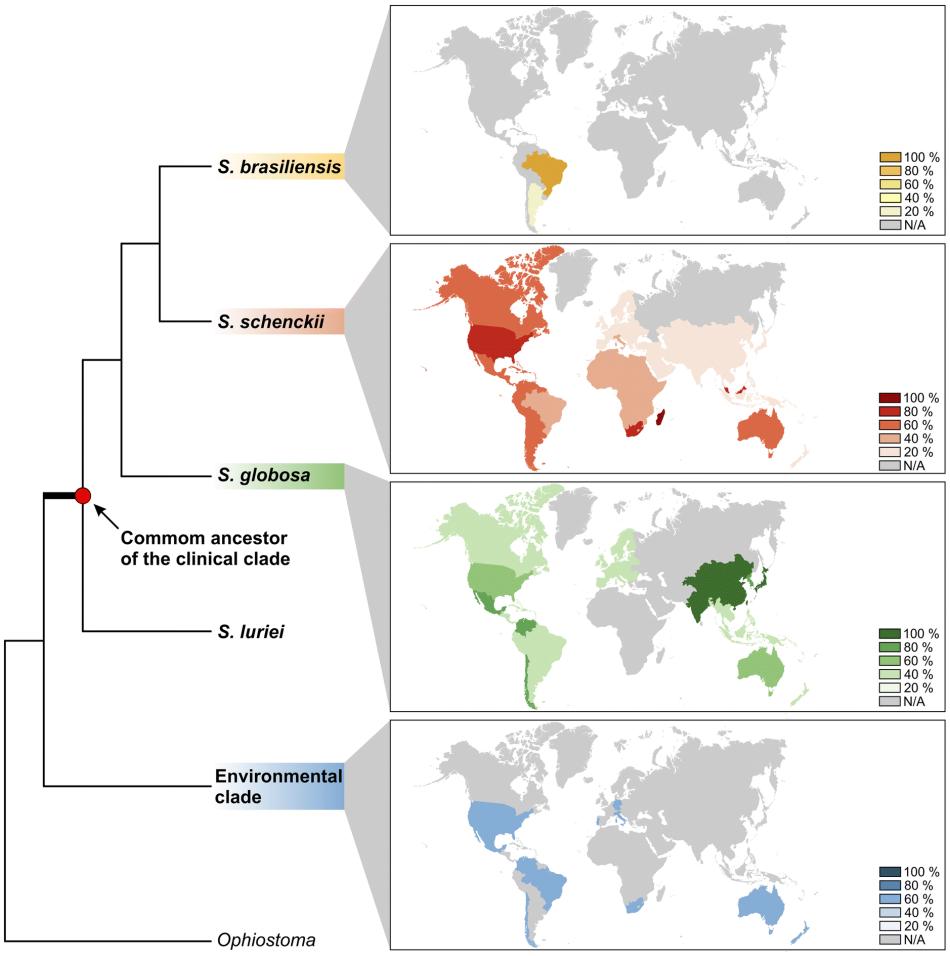


Figure 2. Recent global distribution of *Sporothrix* spp. from the pathogenic (*S. brasiliensis*, *S. schenckii*, and *S. globosa*) and the environmental clade of the *Sporothrix* species. *S. schenckii* has almost worldwide distribution, while *S. brasiliensis* is restricted to the South and Southeast of Brazil. *S. globosa* is found less frequently in the Americas and Europe but is an emerging species in Asia. Sporotrichosis caused by the environmental clade is less frequent but spreads worldwide. Image adapted from Rodrigues et al., 2020.

Clinical sporotrichosis in mammals results primarily from either animal transmission (zoonoses) or plant origin (sapronoses). Until the mid-1990s in Brazil, sporotrichosis was a rare sapronoses caused by *S. schenckii* that appeared only in sporadic, self-limiting clusters. It was an occupational disease that occurred when workers were exposed to a high incidence of injuries with contaminated materials, like the rest of the world (30). In the last decades, however, feline sporotrichosis caused by *S. brasiliensis* has led to a large epidemic with the zoonotic transmission in Brazil, becoming the leading etiological agent of

feline and human sporotrichosis in the region (1,8–10). Nowadays, Brazil is the world's sporotrichosis epicenter, and cases of human sporotrichosis occur in 25 of the 26 Brazilian states. Zoonotic sporotrichosis cases, stated in Brazil's southeast states, have been expanding rapidly toward the northeast regions in recent years. This results directly from the feline sporotrichosis epizootic (1), as illustrated in Figure 3, and a brief timeline of key events of human and animal sporotrichosis is present in Figure 4.

Cats play an essential role in the sporotrichosis epidemic in Brazil. They are very susceptible to the disease, and deep scratching and biting make it highly transmissible to felines and humans (29). Furthermore, treatment of feline sporotrichosis is challenging to the patient, owner, and veterinarian, requiring a long period of daily care, cooperation, persistence, and use of itraconazole in high dosages. The population's poor socioeconomic background and scarce access to human and veterinary health services also worsen the current state of this ongoing epidemic in Brazil (9,15,21). Treatment abandonment is frequent due to the treatment and the long-during struggle to keep infected cats isolated and confined, administrate oral medications, and transport them to the clinic (31,32). Additionally, since factors such as the non-visualization of skin lesions and systemic involvement may lead to misperception of cure, there is a risk of disease recurrence if treatment is discontinued before the therapeutic protocol established by the veterinarian (31).

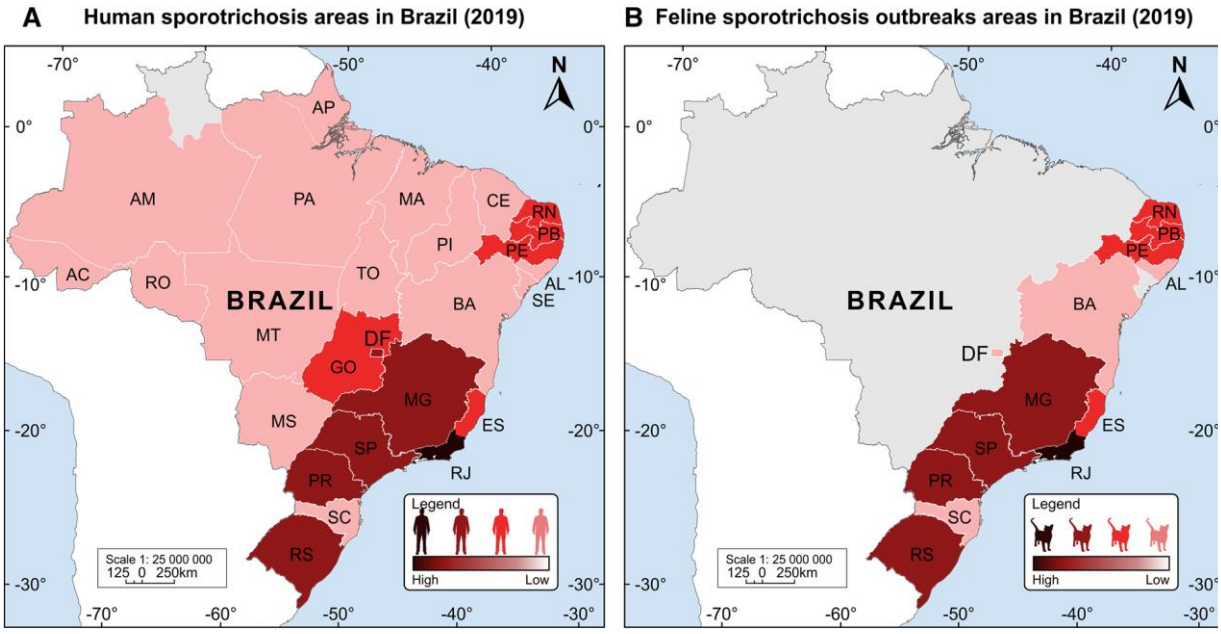


Figure 3. Brazil's geographic distribution of human and feline sporotrichosis in recent years: (A) Cases of human sporotrichosis have been reported in 25 of 26 Brazilian states, with significant differences in frequency. (B) Brazil's South and Southeast states show the world's largest

epizootic feline sporotrichosis. The zoonotic sporotrichosis driven by *S. brasiliensis* is expanding rapidly in Northeast Brazil. Image from Rodrigues et al., 2020.

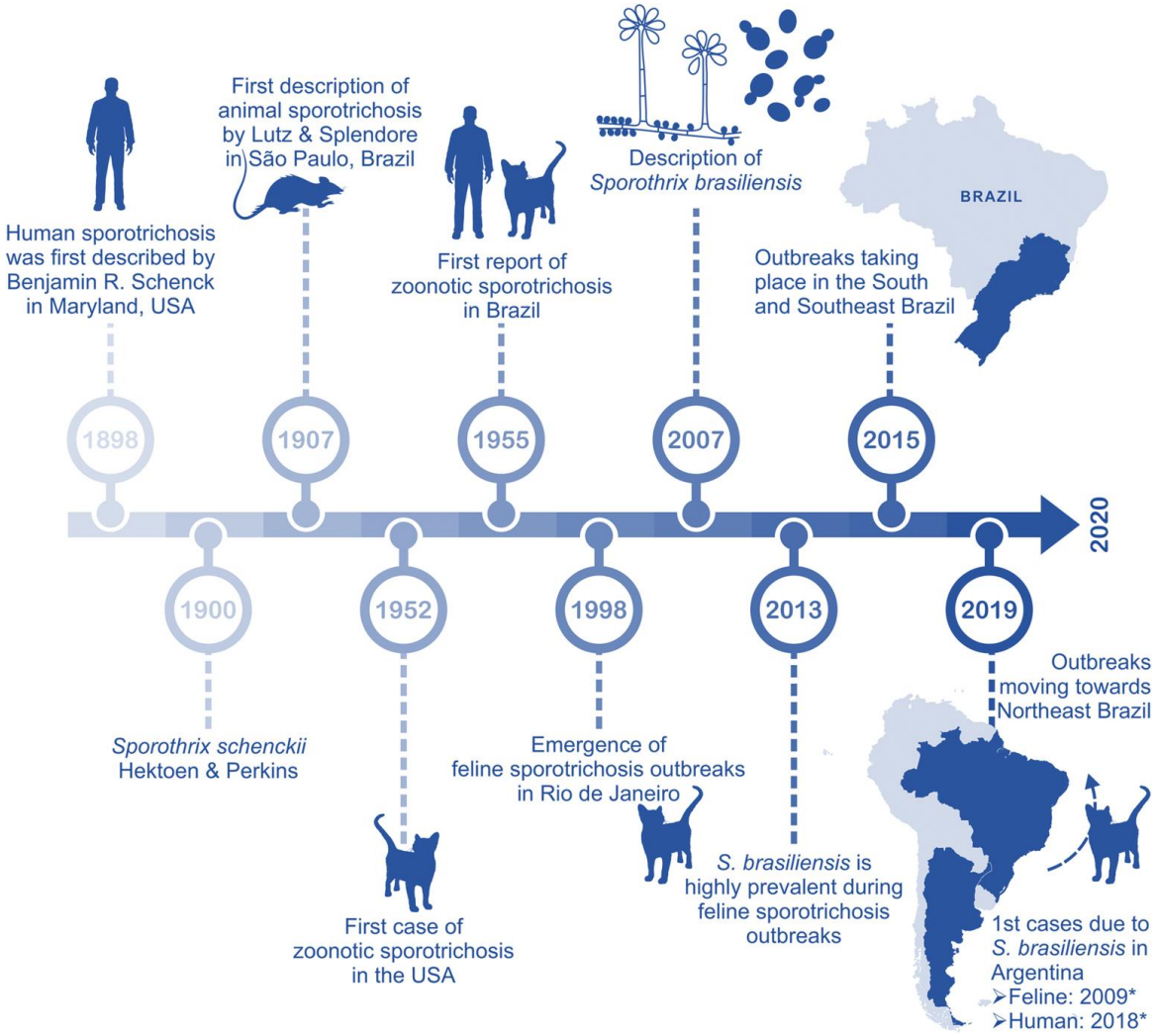


Figure 4. Timeline of key events of human and animal sporotrichosis. In recent decades (1998–2020), *S. brasiliensis* emerged as the primary agent during feline sporotrichosis epizootic and zoonotic transmission in southern and south-eastern Brazil. Image from Rodrigues et al., 2020.

In 2011, 4,100 cases in humans and 3,800 cases in cats were treated in only one institution *Fundação Oswaldo Cruz* (10), in Rio de Janeiro. For this reason, in recent years, sporotrichosis has been considered an insidious disease in the state of Rio de Janeiro, Brazil (33,34), where *S. brasiliensis* was confirmed as the most prevalent species after phenotypic analysis of 247 samples collected (10). Similar epidemics are occurring in the states of São Paulo and Rio Grande do Sul (8). However, the actual prevalence of the disease is undoubtedly underestimated, mainly due to the absence of data from stray feline cases. Even though sporotrichosis has become a mandatory notifiable disease in some Brazilian

states like Minas Gerais, the information about contact with sick animals is still disregarded (35). Urban areas with high feline population densities seem important drivers of epizootics *S. brasiliensis* transmission. The epidemiological profile group usually is people with poor socioeconomic conditions with direct and frequent contact with these animals, namely children, the elderly, and women. Outside urban areas, the classical sapronoses transmission type prevails (8,36). Sporotrichosis has become a public health problem in Brazil, threatening animal owners and veterinarians (1). The fight against sporotrichosis requires the engagement of human and animal health policies to reduce the transmission chain of *Sporothrix* (15). Also, public actions to promote the availability of free veterinary services and antifungal treatment for the pet population, as well as better programs for control of wandering animals and correct destination of animals that come to death due to sporotrichosis, would improve disease control (37). Cytopathological examination or other preliminary diagnostic tests with good sensibility can also be helpful since early feline sporotrichosis treatment contributes to disease control in the community (38).

In addition to the epidemic associated with cats in Brazil, a variety of other animals have been identified as contributing to sporotrichosis in humans and others that may suffer from this disease, especially mammals (39). In Rio de Janeiro, the free provision of public veterinary services and drug supplies is limited to Clinical Research Institute Evandro Chagas (*Instituto de Pesquisa Clínica Evandro Chagas - IPEC*), which is unable to meet the demand for all cases (40). Unfortunately, even with free clinical and laboratory evaluations, treatment abandonment happens with high incidence in feline sporotrichosis cases and may represent a problem in controlling this disease (40). Despite all efforts, until this day, there is no immunoprophylaxis or immunotherapy available to humans or cats against *Sporothrix* spp (41).

Lastly, there is a possibility that an increased virulence of *S. brasiliensis* may be a factor contributing to the sporotrichosis outbreak in Brazil. Experimental models have been used to investigate and bring attention to differences in pathology and virulence factors, where *S. brasiliensis* usually display a higher fungal burden, invasiveness, and extensive tissue damage compared to others (15).

Genetic engineering tools

Cell transformation occurs when exogenous DNA is incorporated into a cell. It is an essential tool to link genes to functions and, consequently, better understand one microorganism's pathophysiology (42). Protoplast-mediated transformation (PMT) is the most common method to transform fungi. However, creating a fungi protoplast requires the removal of its cell wall, whose composition varies between fungi species. Consequently, this technique becomes laborious, which involves standardization

and optimization for the cell wall removal protocol for each fungal species used (43). Since then, other less harsh genetic engineering techniques have been discovered and standardized in several fungi species. The *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique removes this process altogether since the desired gene sequence can also be inserted into intact cells (44), thus avoiding the laborious protoplasts preparation process.

Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens is a phytopathogenic gram-negative bacterium used in several research fields (45). Naturally, the T-DNA inside the Ti plasmid of the bacterium encodes enzymes that influence the production of plant growth regulators, resulting in crown gall disease among most of the plant families inside the plant kingdom (46,47). With the manipulation of its Ti plasmid, this species became a central genetic tool in diverse fields of biological and biotechnological research (45).

ATMT was first designed as a genetic engineering technique to randomly insert the desired DNA sequence into the genome of plants (48). However, under laboratory conditions, the *Agrobacterium* range of eukaryotic organisms became much broader (48), transforming yeasts, filamentous fungi, cultivated mushrooms, cultured human cells, and even *Sporothrix schenckii* (49–54). Although the use of this technique in *S. brasiliensis* is scarce (55), it has been standardized in our laboratory by collaborators.

Plasmids containing the desired T-DNA are constructed and inserted into the *A. tumefaciens*, which then insert this portion of DNA into the host's genome, as schematized in Figure 5. The T-DNA is often inserted in fungi by homologous recombination, allowing for targeted integration of the foreign DNA if it shares sequence homology with the host genome. This provides genomic tools for insertional mutagenesis or specific gene replacement (48). Moreover, the non-sequence-specific way Agrobacterium integrates its T-DNA within the host genome produces many transformants when transforming intact cells, such as conidia, mycelium, and yeasts (51). This technique has already been standardized in several thermally dimorphic fungi (56–62), including *Sporothrix schenckii* (49–51). However, the use of this technique in *S. brasiliensis* is scarce (55). ATMT provided new insights into fungal pathogenesis, pigmentation, sporulation, and antibiotic resistance (51) and can also help enlighten the virulence mechanisms of *S. brasiliensis.*

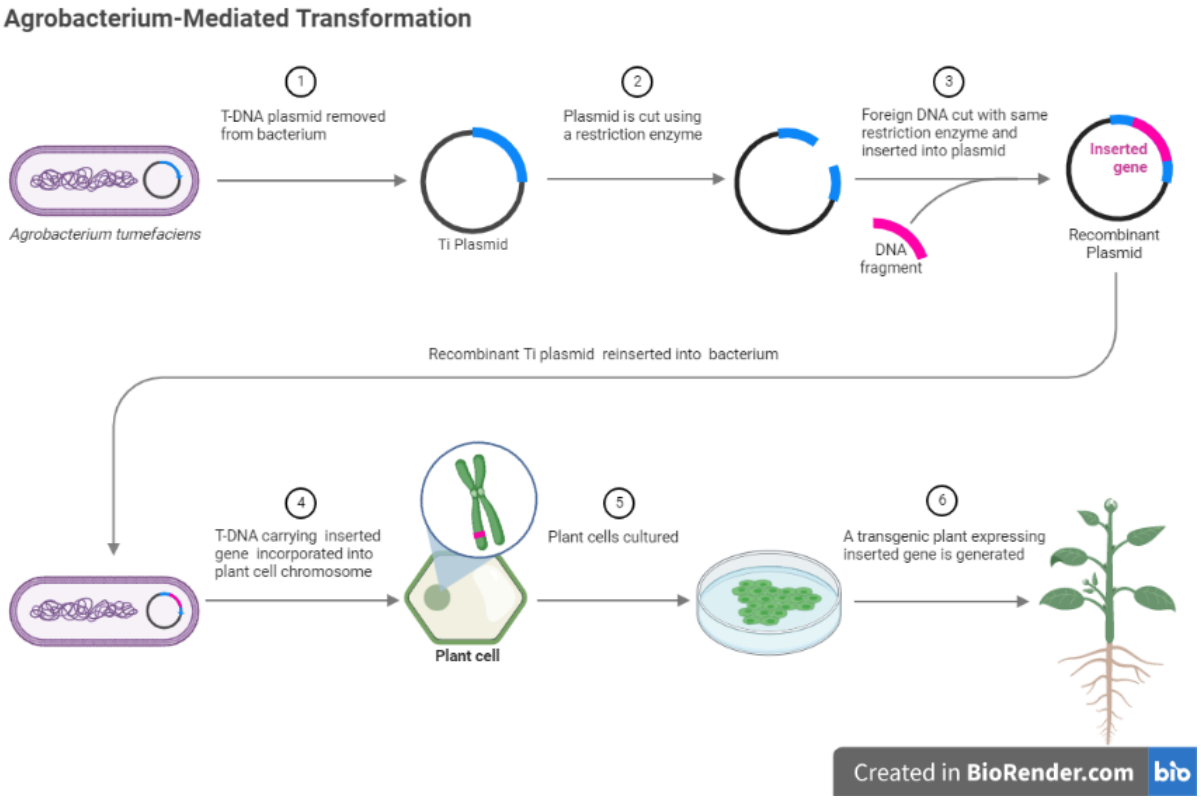


Figure 5. The schematization of the ATMT technique in plants.

CRISPR/Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems were discovered as a bacterial and archaeal endogenous immune/defense mechanism against invading viruses, in addition to other unrelated functions, such as gene regulation (63). There is a great natural diversity of CRISPR/Cas systems in archaeal and bacterial systems, with several target preferences according to the type of proteins and RNA components. The system can target only DNA, RNA, or both the DNA and RNA (64–66). During the last years, however, this natural system has been applied to different organisms, from bacteria to human cells, to target genomes aiming for gene editions (67). CRISPR/Cas9 system is an easy and efficient programable genome editing tool capable of modifying genes in a broad spectrum of cell types (68), such as cancer cells (69) and fungal pathogens (67,70).

The CRISPR-Cas9 system has two components, the Cas9 nuclease and a single chimeric guide RNA (sgRNA), which guides the nuclease protein to the locus of interest by base pairing, generating a double-stranded DNA break (67,70). The continuous cleavage of the DNA by the targeted Cas9 nuclease will ultimately promote small base insertions or deletions by the host nonhomologous end joining repair system, generating a loss of gene function through the synthesis of frameshifted nonsense proteins (70), as exemplified in Figure 6.

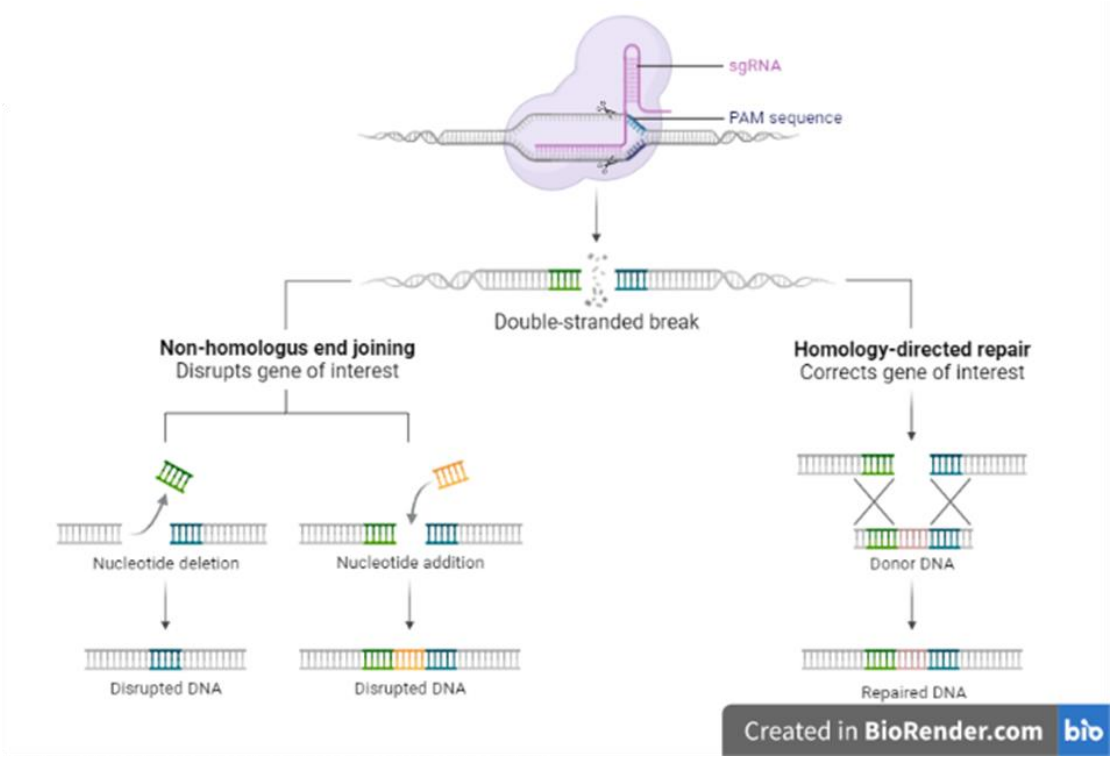


Figure 6. CRISPR/Cas9 Gene editing model.

Recently, the emergence of the CRISPR/Cas systems has become a pillar of experimental biology with a convenient implementation, high mutation efficiency, and great potential for therapeutic applications (71). However, since the off-targeted system can result in cell toxicity, further studies and optimizations for each biological system are required (72). Although off-target after CRISPR-Cas9 mutagenesis may not be a big problem for filamentous fungi, it is still advisable to keep cas9 and sgRNA expression to a minimum (67). The CRISPR/Cas9 system has already been applied in several fungi, such as *Aspergillus* spp., *Candida* spp., and *Saccharomyces cerevisiae* (73–76).

Fluorescent Proteins

The limited repertoire of genetic manipulation tools for the *Sporothrix* genus still limits research *in S. brasiliensis* pathophysiology (77,78). Autofluorescence is the natural emission of light by biological structures arising from endogenous fluorophores, primarily from mitochondria and lysosomes when excited by UV/Vis radiation of a suitable wavelength (79).

Fluorescent proteins emit light far beyond the intensity of endogenous cellular fluorophores. When used, they essentially provide a 'fluorescent tag' on a desired protein or structure, making it possible to probe its location, activity, or interaction with other molecules from a subcellular to a multicellular scale (80,81). Consequently, fluorescent proteins are a powerful and convenient tool that assists research and

experimentation with intact living cells and organisms in several biological fields (82,83). Their use has already been reported on a broad spectrum of cells like protozoa, fungi, plants, animals, and viruses (84–88).

Green fluorescent protein (GFP), discovered in the bioluminescent jellyfish *Aequorea victoria*, can be expressed in either eukaryotic or prokaryotic cells. It emits a bright green fluorescence easily detected by various instrument platforms, independently of any cofactors, substrates, or gene products from *A. Victoria* (80). The mCherry protein is a fluorescent derivative protein originating from the monomer mRFP1, a non-toxic and non-disruptive version of the fluorescent protein DsRed, found in the *Discosoma* spp. (89). The mCherry protein emits a bright red fluorescence and, like GFP, can act as an intracellular probe and have *in vitro* and *in vivo* applications (90). Most proteins tagged by the GFP and the mCherry protein retain regular biological activity (80,89).

Transport of all proteins in a eukaryotic cell begins in the cytosol, but organelle proteins are then selectively and efficiently transported into their destination compartments to exercise their physiological functions (91). Short peptide sequences called localization signals act as signal fragments, mediating their transport from the cytoplasm into the targeted region (91,92). These molecules, such as can nuclear and mitochondrial localization signals (NLS and MLS, respectively, can be merged with fluorescent proteins to conduct them into the desired location inside the cell, essentially allowing the creation of transformants with localized fluorescence (91,92).

CHAPTER 2 – Aims and Outline

The mechanisms involved in the increased virulence of *Sporothrix brasiliensis* remain unclear. Gene editing techniques are an essential tool that allows the identification of molecular mechanisms and cellular processes that may be directly involved in the microorganisms' virulence, thus, can clarify the role of gene functions in vitro and in vivo. The design and creation of plasmids that allow the making of transformants with fluorescent proteins and the application of techniques such as CRISPR/Cas9 are essential mechanisms that would enable the study and understanding of several factors that are still unknown in microorganisms. Therefore, our main objectives were:

1. To construct plasmids to perform the CRISPR/Cas9 technique in *Sporothrix brasiliensis*.
2. To design, create and categorize *S. brasiliensis* tagged strains with fluorescent proteins.

The work presented in this thesis aims to contribute to developing molecular tools to unravel the mechanisms of *S. brasiliensis* virulence.

Chapter 1 introduces the state of the art of sporotrichosis regarding its etiological agents, epidemiology aspects, and distribution throughout the world. Secondly, a concise description of gene editing mechanisms, such as CRISPR/Cas9 and ATMT techniques, and a brief introduction to fluorescent proteins and their use in biology.

Chapter 2 describes the objectives of this dissertation and its design.

Chapter 3 focuses on the optimization and construction of plasmids for performing the CRISPR/Cas9 technique in *Sporothrix brasiliensis*. Although CRISPR/Cas9 constructs targeting the SPBR_01718 gene were inserted in the *S. brasiliensis* genome, no uracil auxotrophic mutants were obtained. Our data show that the TEF1-α promoter was unable to drive Cas9 expression.

Chapter 4 focuses on the creation of a genetic toolbox for *S. brasiliensis*. Firstly, fluorescent-tagged *S. brasiliensis* strains were produced, with green (GFP) or red (mCherry) fluorescence. The expression of these proteins allowed the study of two different endogenous *S. brasiliensis* promoters, namely pGAPDH and pH2A, and their expression profile. Secondly, easy-to-edit plasmids were built, allowing for the expression of the in-frame fusion of proteins of interest with either mCherry or GFP. Lastly, several tag strains were created and analyzed microscopically regarding their expression of GFP or mCherry fluorescent proteins, with different location signals, guiding them to the cytosol or nucleus.

Chapter 5 presents a broad discussion and future perspectives regarding this thesis.

CHAPTER 3 – CRISPR/Cas9 gene editing system in *Sporothrix brasiliensis*

Abstract

Sporotrichosis is the world's most prevalent and distributed subcutaneous mycosis. *S. brasiliensis* species is reported as the most virulent species, exhibiting the worst clinical manifestations, but the infection and virulence mechanisms of *Sporothrix* spp. are still not clearly understood. Therefore, the development of genetic engineering techniques for *S. brasiliensis* transformation is urgently needed, such as CRISPR/Cas9 and ATMT. Aiming at selection of uracil auxotrophic mutants, the minimal inhibitory concentration of the positive counter-selection marker 5-fluorouracil-6-carboxylic acid (5-FOA) in *S. brasiliensis* was tested. For transformant selection, during the *Agrobacterium tumefaciens*-Mediated Transformation technique, we used the higher concentration of 0.3% (w/v) 5-FOA and 150 µg/mL of hygB to avoid unspecific growth. The auxotrophic selection marker was intended to select transformants whose SPBR_01718 gene had been cleavage by the CRISPR/Cas9 system. Although CRISPR/Cas9 constructs targeting this gene were inserted in the *S. brasiliensis* genome, confirmed by the PCR technique through the *HPH* gene, no uracil auxotrophic mutants were obtained. As so, we analyzed the expression of the CRISPR/Cas9 system in *S. brasiliensis* with the Cas9 endonuclease tagged to the fluorescent protein mCherry, but no fluorescence was detected in microscopic analysis, thus confirming the absence of Cas9 protein expression. The pTEF1-α has already been reported as a suitable promoter to express the CRISPR/Cas9 system in filamentous and dimorphic fungi. However, our results suggest that the TEF1-α constitutive promoter, present in the pPTS608 plasmid used, previously designed for *Blastomyces dermatitidis*, could not drive Cas9 expression in the *S. brasiliensis* biological system.

Introduction

Techniques to select mutant cells in the presence of large numbers of wildtype cells are a handy tool in diverse fields of biological and biotechnological research (93). These selective techniques usually rely on the mutants' ability to convert and inactivate a compound that would otherwise be toxic to wildtype cells. Selection markers, such as hygromycin B and chlorimuron Ethyl, are examples of toxic compounds that fungi mutants harboring, respectively, the HPH and SUR genes can inactivate (94–96). Auxotrophic selection markers are inert compounds only wildtype cells can interact with to synthesize a toxic compound, ultimately inhibiting their growth. Since auxotrophic mutant cells cannot form these poisonous compounds, they can grow in inert precursors' presence (93,97). These strains usually carry mutations in biosynthetic genes, typically genes needed for amino acid or nucleotide biosynthesis (97). The inert precursor 5-Fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate; 5-FOA) is used in yeast molecular genetics to identify the expression of the URA3 gene, which encodes orotine-5'-monophosphate

(OMP) decarboxylase (98). Yeasts with an active URA3 or similar gene (Ura+) convert 5-FOA to fluorodeoxyuridine, which is toxic to cells. The URA3- cells, or SPBR_01718- in the case of *S. brasiliensis*, grow in the presence of 5-FOA if the media is enriched with uracil. The 5-FOA is frequently used to select uracil auxotrophic mutants, as exemplified in *Saccharomyces cerevisiae* (URA3), *Schizosaccharomyces pombe* (URA4 and URA5), *Candida albicans* (URA3), *Aspergillus* spp**.** (99), and *E. coli* (pyrF) (93,98).

The sequenced genomes database is rapidly increasing, and techniques such as CRISPR-Cas9 adapted for use in filamentous fungi help to understand their biology (67). In the fungal pathogen *Blastomyces dermatitidis,* a CRISPR/cas9 system was developed, taking advantage of the plasmid pPTS608_Cas9_hyg that mediates Cas9 and sgRNA expression (70). This CRISPR-Cas9 gene editing system involves the activity of ribozymes to generate the active RNA portion (sgRNA), and when co-expressed with Cas9, the target sequence is modified (70). CRISPR/Cas9 is a powerful genetic editing tool with several applications. It allows a better understanding of different mechanisms related to aspects of a particular gene and its relationship with cellular mechanisms. Developing CRISPR-Cas9 techniques in *S. brasiliensis* would be necessary as a gene-editing tool that can help uncover and enlighten underlying virulence mechanisms.

In the present study, we evaluated the application of the CRISPR/cas9 system developed in *S. brasiliensis*.

Material and Methods

Microorganisms and culture media

The *S. brasiliensis* ATCC MYA-4823 strain (55,100) was used throughout this study. Cells were cultured at 37°C for 72h in yeast extract peptone dextrose (YPD, pH = 7.8). Yeast cells were obtained through sterile gaze filtration to remove cell aggregates and hyphae. The *A. tumefaciens* strain AGL-1 (101,102) strain, kindly donated by Dr. Augusto Schrank (Federal University of Rio Grande do Sul, Brazil), harbored each plasmid used in the experiments. *A. tumefaciens* cells were cultured at 26°C in L.C. BROTH medium, supplemented with the antibiotic rifampicin (20µg/mL, Sigma-Aldrich©). To selective medium used kanamycin (100µg/mL, Formedium™) to maintain the plasmids used in this work. The L.B. medium, supplemented or not with kanamycin (50µg/mL), was used for *E. coli* growth at 37°C, while the SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) liquid medium was used to allow recovery of *E. coli* in the *in vivo* Assembly technique (103). Each culture medium component is described in Table 1. Cultures were maintained at 4°C for short-term storage and were regularly sub-cultured every 4–6 weeks.

Table 1. Culture media used in this study.

YPD		LB		LC	
Dextrose	2.0%	**NaCl**	1.0%	**NaCl**	0.8%
Peptone	2.0%	**Tryptone**	1.0%	**Tryptone**	1.0%
Yeast extract	1.0%	**Yeast extract**	0.5%	**Yeast extract**	0.5%
***Agar**	1,5%	***Agar**	1.5%	***Agar**	1.5%

***Agar was added only in solid medium.

Agrobacterium tumefaciens transformation protocols

For electrocompetent cells of *Agrobacterium tumefaciens* and electroporation, adapted protocols were used. *A. tumefaciens* cells were grown at 28°C to an optical density (OD_{660nm}) of 1.5-2.0. Collected by centrifugation and washed five to seven times in sterile distilled water. Later, the cells were resuspended in 10% glycerol to a final concentration of $1x10^{10}$ cells/ml and stored frozen at -70°C. Cells were defrosted on ice, and 2ng of plasmid DNA was added to a 40µl aliquot of electrocompetent cells. The plasmid and cells were homogenized by pipetting gently, and the mixture was transferred to a cooled cuvette. For electroporation, the Gene Pulser Xcell TM (Bio-rad, model 1652660) was used with: 1.8kV, 200Ω, and 25µF. Following electroporation, 900µl of L.B. is added to the cuvette and transferred the total volume to a sterile tube, where it is incubated at 26°C without shaking for 3 hours. After incubation, the volume is centrifuged, plated, and again incubated at 26°C in a selective solid medium with kanamycin (100µg/ml) for 72 hours (104).

The *A. tumefaciens*-mediated transformation (ATMT) method was based on the previously described *Sporothrix* spp. protocols with adjustments (49–51,55), and schematized in Figures 7 and 8. *A. tumefaciens* cells previously transformed by electroporation were cultured at 28°C while shaking (200 rpm) in 10 mL of L.C. broth with antibiotics for 6-10h. Then centrifuged at 4000 rpm for 6 minutes and resuspended in Induction Medium (I.M.) (46) with rifampicin (20µg/ml), kanamycin (100µg/ml), and acetosyringone (200µM) to an OD_{660nm} of 0.3.

In the I.M. medium, the *A. tumefaciens* cells were grown overnight at 26°C, 200 rpm, for an OD_{660nm} of 0.6-0.8 to virulence. Simultaneously, exponential growth fungal cells were grown at 37°C, 200 rpm, for 24h in 50mL YPD (pH 7.8), and yeast cells were obtained through filtration with a sterilized gaze. *A. tumefaciens* was mixed with *S. brasiliensis* cells in a 2:1 ratio. $5x10^7$ cells of *S. brasiliensis* and $1x10^8$ of *A. tumefaciens* cells were used. Those mixtures were centrifuged and inoculated onto I.M. plates on a sterile membrane (Hybond™-C). The plates were co-cultivated in the dark for 72 hours. After the co-

cultivation period, the membranes were transferred to falcon tubes. The cells were dislodged into a 5mL YPD medium (pH 7.8) containing cefotaxime (200µg/mL) for growth inhibition of *A. tumefaciens*.

After 6 hours of cultivation at 37°C, while shaking at 200 rpm, 1mL was centrifuged, about 800µL supernatant was removed, and the final volume inoculated on a selective YPD solid medium (hygromycin B, 150 µg/mL) (55). Fungal cells without the *A. tumefaciens* were inoculated into selective and non-selective YPD mediums as positive and negative controls. To conclude, after cultivation for a week at 37°C, the transformants were randomly selected for other experiments.

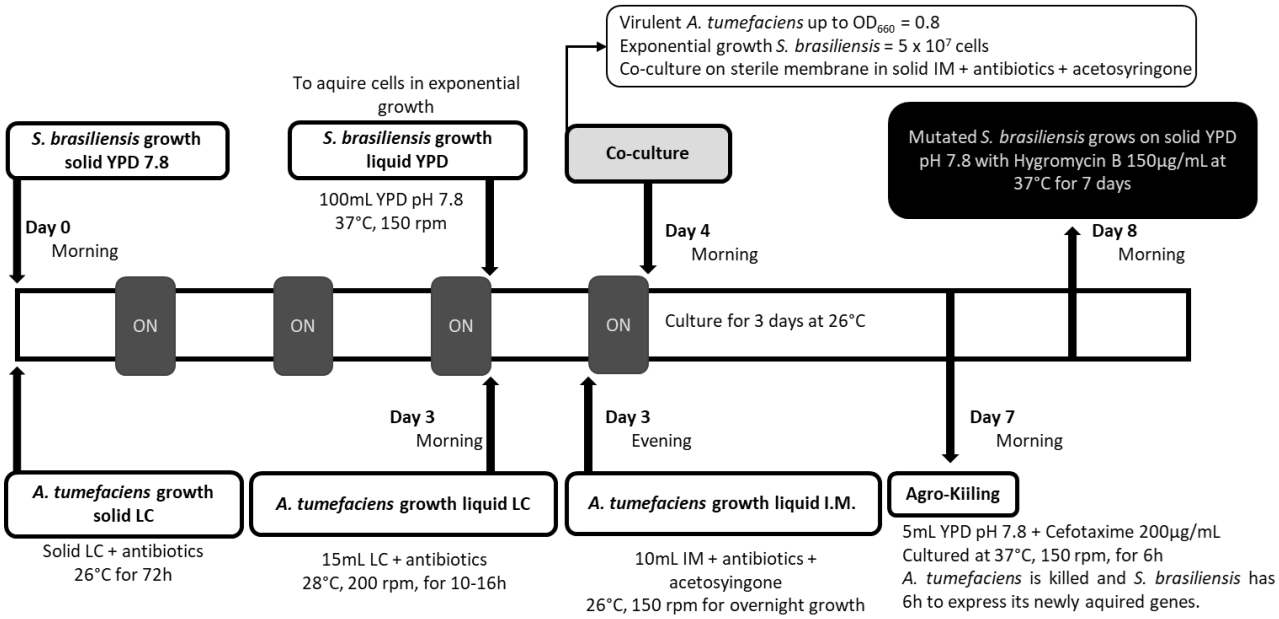


Figure 7. Timeline schematic of *S. brasiliensis* ATMT.

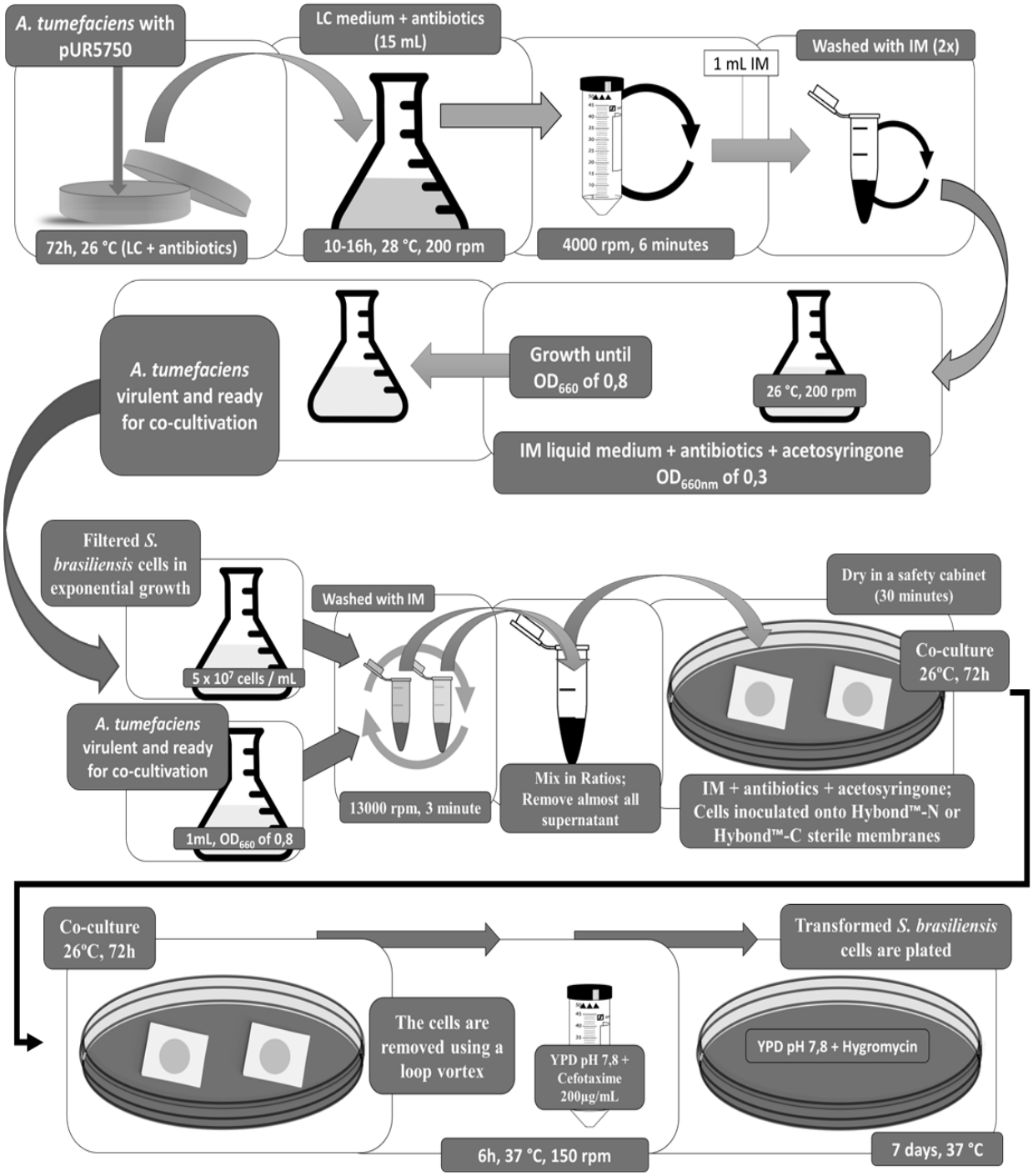


Figure 8. Schematic of *S. brasiliensis* ATMT technique.

The 5-Fluoroorotic Acid (5-FOA) inhibitory concentration in *S. brasiliensis*

To identify the inhibitory concentration of 5-FOA in *S. brasiliensis* ATCC 4823, cells were obtained in YPD liquid medium pH 7.8 and diluted to an optical density of 1, measured at 660nm (OD_{660nm}) by the Genesys™ 20 Spectrophotometer (Sigma-Aldrich, model Z376035). The sample was diluted in three different cell concentrations in milliliters (10^6 cells/mL, 10^5 cells/mL, and 10^4 cells/mL). After dilution,

the samples were inoculated in plates containing solid medium YPD pH 7.8, with different 5-FOA concentrations. One drop (5µL) of each concentration was inoculated for each plate: 10^6 on the left edge, 10^5 on the middle edge, and 10^4 on the right edge.

Plasmid Construction

CRISPR/Cas9 targeting SPBR_01718 gene

To apply the CRISPR/Cas9 gene editing technique to the fungi *S. brasiliensis*, the vector pPTS608-Cas9-HygB was used as the backbone, and pPTS608-Cas9-HygB-PRA1-sgRNA for the amplification of the fragments to the sgRNA and desired protospacers sequences, as described elsewhere (70). In this strategy, two protospacers to disrupt the SPBR_01718 gene (Figure 9), which encodes the uridine monophosphate synthetase in *S. brasiliensis*, were selected using the CHOPCHOP web software (105). Through a blast of the sequences chosen as protospacers, it was possible to verify that the same sequences could be used for the same purpose in the fungus *S. schenckii*, probably due to their genetic proximity.

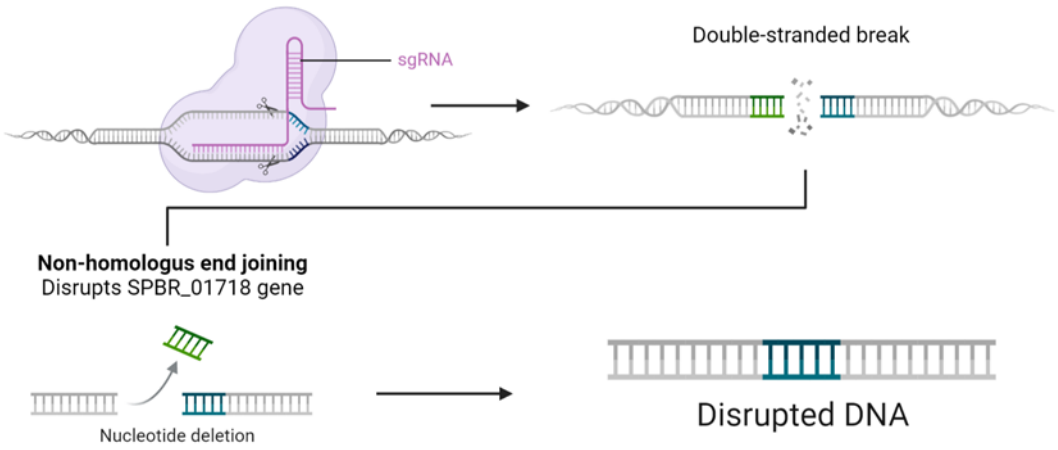


Figure 9. CRISPR/Cas9 gene-editing schematic in S. brasiliensis. Representation of CRISPR/Cas9 system to disrupt the SPBR_01718 gene.

As mentioned, the amplification of the fragment containing the chosen protospacers and the sgRNA, the plasmid PRA1 was used. The fragments insert the desired protospacer into the sgRNA and have sequences complementary to the plasmid pPTS608-Cas9-HygB after linearizing with the PacI enzyme, as represented in Figure 10.

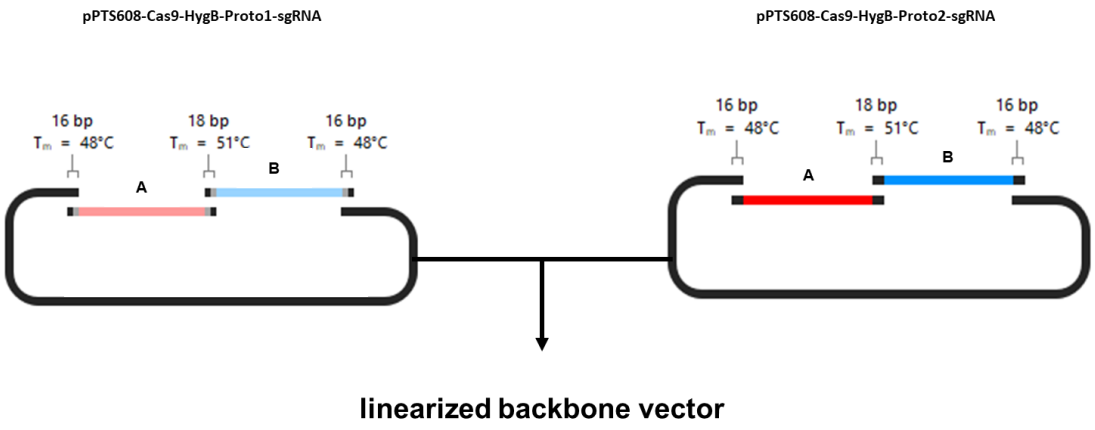


Figure 10. **Backbone vector pPTS608-Cas9-HygB** linearized with PacI enzyme and pPTS608-Cas9-HygB-Proto1-sgRNA and pPTS608-Cas9-HygB-Proto2-sgRNA with their respective fragments.

Fragments were created to construct plasmid pPTS608-Cas9-HygB-Proto1-sgRNA (Vector backbone + sgRNA with protospacer 1). Fragment A uses the primers Proto_1_ Frag A R, which inserts the mutation in the hammerhead, and the primer C9_Frag A F, which amplifies o part of the sequence referring to the sgRNA. Fragment B uses the primers Proto_1_Frag B F, which inserts protospacer 1 into sgRNA, and the C9_Frag B R, which ensures amplification of the rest of the sequence that has binding sites with the backbone plasmid. For the plasmid pPTS608-Cas9-HygB-Proto2-sgRNA (Vector backbone + sgRNA with protospacer 2), two fragments were also created, fragment A - uses primers Proto_2_ Frag A R, which insert mutation in the hammerhead + primer C9_Frag A F, and fragment A – uses primers Proto_2_Frag B F, which inserts the one that inserts protospacer 2 into sgRNA + the primer C9_Frag B R. The construction of the final vectors was carried out through the Gibson assembly (106). The *E. coli* NZYStar Competent Cells (NzyTech™, Portugal) were used, and the appropriate transformation protocol was followed. The protospacers sequences are represented in Table 2.

Table 2. Protospacers, orientation, genome context and PAM sequence.

PROTOSPACER 1			
Orientation	**Protospacer sequence**	**Context Sequence***	**PAM Sequence**
antisense	ATGATGCGGGCCGTACCGG	ACTCAATGATGCGGGCCGTACCGGCGGTGT	CGG
PROTOSPACER 2			
Orientation	**Protospacer sequence**	**Context Sequence***	**PAM Sequence**
sense	CTACGACCTTGTCACCGGAT	CCCACTACGACCTTGTCACCGGATGGGACT	GGG

*Protospacer sequence in *S. brasiliensis* genome. In blue the sequence refers to the protospacer, while the red sequence illustrates the PAM sequence.

The fragments were created by PCR amplification with primers containing the 20 nucleotides (nt) protospacer and the 6 nt inverted ribozyme repeat overlapping the end of the protospacer, each targeting vector must replace 20 nt protospacer and 6 nt inverted hammerhead repeat required to maintain sequence complementarity (70), the primers are shown in Table 3.

Table 3. Primers used in the CRISPR/Cas9 strategy.

Primer name	Sequence	nt
C9_Frag A F	ccgctgagggtttaatGCGTAAGCTCCCTAATTG	34
C9_Frag B R	cggctgaggtcttaatGAGCCAAGAGCGGATTCC	34
Seq UMS_L	TCCCACCATATCCTCTCGTC	21
Seq UMS_R	CCGTCCTGGTGGTAGGTG	18
Proto_1_Frag B F	GAAACGAGTAAGCTCGTCAATGATGCGGGCCGTACCGGGTTTTAGAGCTAGAAATAGC	60
Proto_1_ Frag A R	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAATGATCGGTGATGTCTGCTCAAG	61
Proto_2_Frag B F	GAAACGAGTAAGCTCGTCCTACGACCTTGTCACCGGATGTTTTAGAGCTAGAAATAGC	60
Proto_2_ Frag A R	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCTACGACGGTGATGTCTGCTCAAG	61
cas9_mcherry 1	GAAAAGAATCCCATCGACTTTC	22
cas9_mcherry 2	GGTGGCGACCGGTGGATCCGTGCTTCCCGAGACCTTGCGCTTCTTCTTG	49
cas9_mcherry 3	TCGGGAAGCACGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGA	50
cas9_mcherry 4	CAGAACTCTGTCCAGGTCATGTAATATCACACGCAGCCGAAGCCTTCGAG	50

The sgRNA cassettes were generated by overlapping high-fidelity polymerase PCR-generated fragments, inserted into the vector backbone by Gibson assembly reaction, and confirmed by Sanger DNA sequencing (70). Plasmid DNA extraction, recombinant DNA manipulation, and *E. coli* and *A. tumefaciens* transformation procedures were performed as reported previously (104,107). *Sporothrix* spp. transformants were selected by hygromycin B resistance. The backbone plasmid is represented in Figure 11.

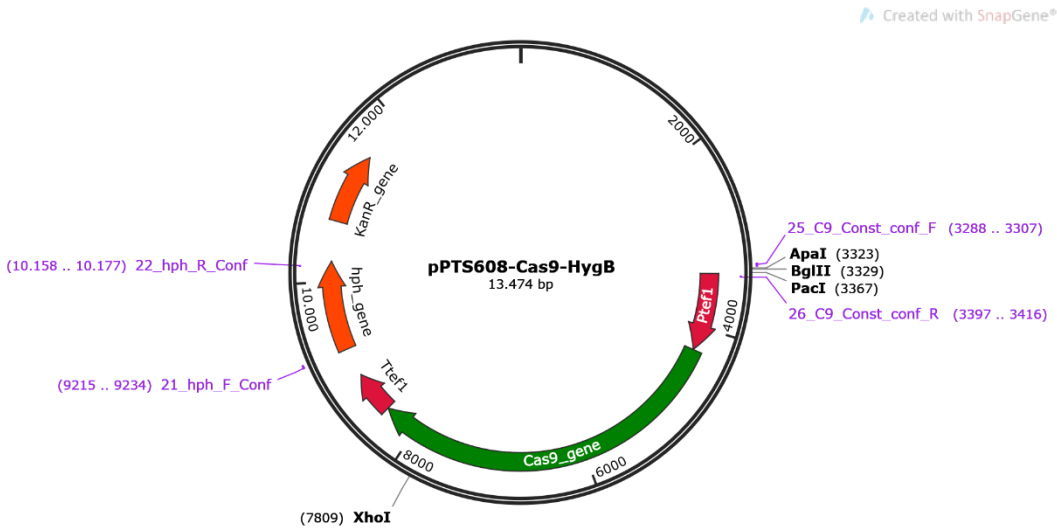


Figure 11. pPTS608-Cas9-HygB map. Cas9 plasmid construction representation with the selection markers hygromycin for *S. brasiliensis* and kanamycin for *A. tumefaciens* and *E. coli*.

pPTS608_Cas9-mCherry

We also designed a plasmid with the Cas9 gene fusion and mCherry fluorescence. For the construction of this plasmid, the backbone vector was digested with the enzymes XhoI and PmlI. And two fragments were designed that contain a linker sequence and the mCherry fluorescence gene. The first fragment was amplified from the backbone vector to insert the linker and a part of the Cas9 sequence that was lost in digestion (primers cas9_mcherry 1 and cas9_mcherry 2). The second fragment was responsible for inserting the linker and the sequence referring to the mCherry fluorescence gene. It was amplified from a plasmid containing the mCherry gene (primers cas9_mcherry 3 and cas9_mcherry 4).

Results and Discussion

5-FOA inhibitory concentration in *S. brasiliensis* ATCC 4823

Aiming to produce uracil auxotrophic mutants using the CRISPR/cas9 strategy, we intend to use 5-FOA as a positive counter-selection marker, as uracil-prototrophic cells will perish (109–111). Three *S. brasiliensis* concentrations (10^6, 10^5, and 10^4) were inoculated into plates (YPD pH 7.8) with different 5-FOA's concentrations. As shown in Figure 12, our data show a gradual growth inhibition at 0.025% (w/v) of 5-FOA at the droplet of 104 cells/mL, and a clear growth inhibition at all the three droplets in the concentrations of 0.3% (w/v) of 5-FOA. Therefore, we selected this higher concentration for further studies

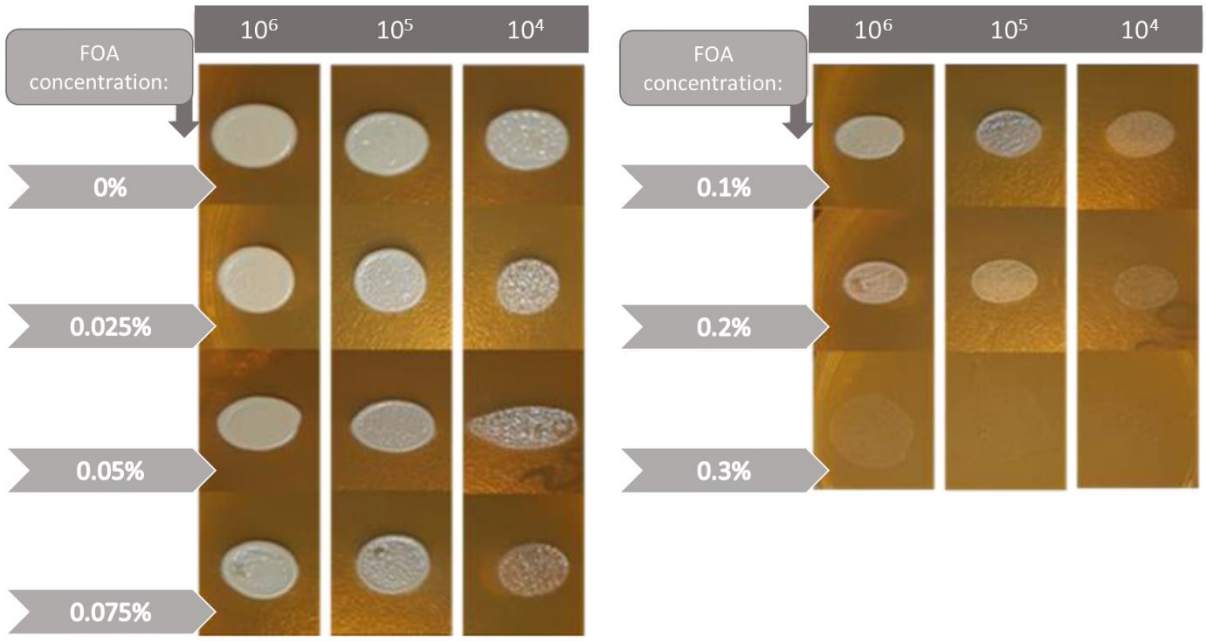


Figure 12. Growth inhibition of *S. brasiliensis* in YPD 7.8 medium with different 5-FOA concentrations (%; w/v). Three droplets with decreasing 10-fold dilutions of ATCC MYA-4823 yeast cells (10^6 cells/mL on the left edge, 10^5 cells/mL in the middle, and 10^4 cells/mL on the right edge) were inoculated in YPD pH 7.8 plates with increasing 5-FOA concentrations.

CRISPR/Cas9 gene editing system

To our knowledge, transformation by CRISPR/cas9 of fungi of the genus *Sporothrix* is yet to be achieved. Considering that the targeted sequence is important for the efficiency of the CRISPR/Cas9 system, we designed within the SPBR_01718 gene two different protospacers (Table 2). The plasmids obtained, pPTS608-Cas9-HygB-Proto1-sgRNA and pPTS608-Cas9-HygB-Proto2-sgRNA, were confirmed by Sanger sequencing (data not shown). With the plasmids successfully constructed, the *Agrobacterium tumefaciens* harboring these plasmids was used in ATMT to transform *S. brasiliensis*.

Firstly, the ATMT was performed using a selective medium with hygromycin B and 5-FOA auxotrophic selection marker (0.3%, w/v) to inhibit the growth of SPBR_01718$^+$ cells (99). However, no colonies appeared at the end of the ATMT's 7-day incubation period. With this data, we thought that no uracil auxotrophic mutants were being produced, or the 5-FOA was toxic for the mutant selection. Therefore, another ATMT was performed and transformed cells were plated in a selective medium with hygromycin B, lacking 5-FOA. After this first round of selection (strains harboring the Cas9 construct), forty colonies were randomly selected and transferred to three different solid culture mediums: minimal medium without uracil, which would only allow the growth of prototrophic mutants (wildtype phenotype; no edition); minimal medium with the addition of uracil, allowing the growth of uracil-auxotrophic and

prototrophic mutants (all transformants); and the permissive YPD medium (that would enable the growth of all mutants).

Our data show that all transformants tested were able to grow in all media, indicating that no auxotrophic mutant was produced. Even so, and as Cas9 cleaves the DNA in the surrounding regions of that protospacer sequence, we decided to sequence the SPBR_01718 gene in two randomly selected mutants. We confirmed by PCR the presence of the HPH gene in these transformants. However, sequencing data surrounding the protospacer regions did not show any genomic alteration in the SPBR_01718 gene, despite the success of the ATMT (Figure 13).

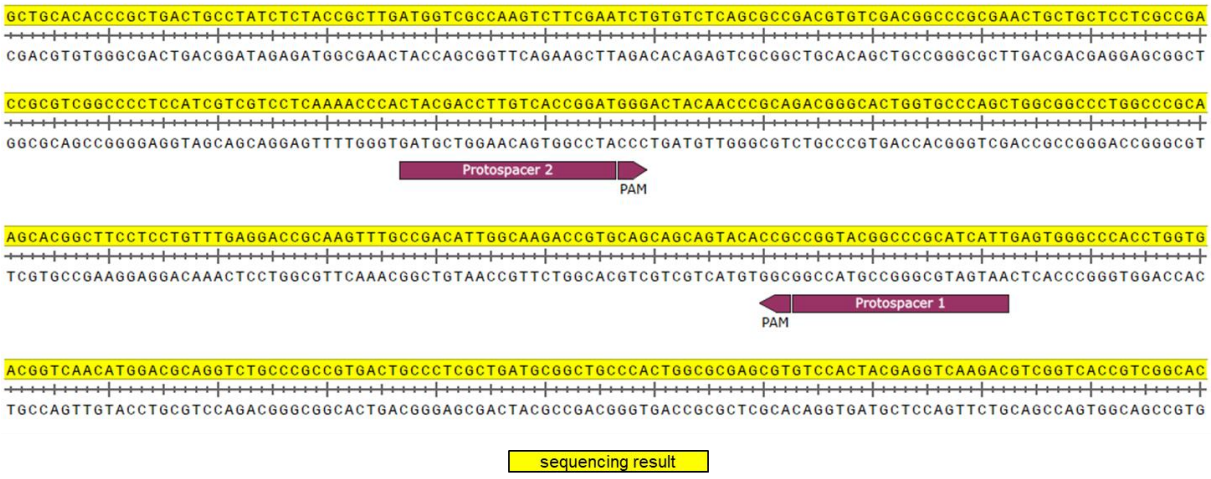


Figure 13. *S. brasiliensis* SPBR_01718 gene alignment. The genetic sequencing of the isolated mutant *Sporothrix brasiliensis* is highlighted and shows no difference from the wild type. For sequencing, primers Seq UMS_L and Seq UMS_R were used.

S. brasiliensis harboring the CRISPR/Cas9 construct targeting the SPBR_01718 gene did not let to any auxotrophic uracil mutants. Considering that, we questioned if the promoter of the translational elongation factor EF-1 alpha (TEF1-α) that drives Cas9 expression in this system, from *Aspergillus* spp., was functional in *S. brasiliensis*. To further evaluate the expression levels of Cas9 and its nuclear localization, essential for its nuclease function, we construct a new plasmid with an *in-frame* fusion of Cas9 with mCherry, spaced by an inert linker – SGSTDPPVA (108). The plasmid pPTS608_Ca9-mCherry was confirmed by sequencing and used for transformation. *S. brasiliensis* pPTS608_Ca9-mCherry mutants were produced through the ATMT technique. Our data show the absence of red fluorescence, confirming the absence of Cas9 protein expression. In a preliminary assay we also could not detect Cas9 expression by western blot analysis using Cas9 antibody (7A9-3A3) from Santa Cruz (data not shown). The pTEF1-α has already been reported as a suitable promoter to express the CRISPR/Cas9 system in

filamentous and dimorphic fungi (67,70). However, our results suggest that the TEF1-α constitutive promoter does not express the CRISPR/Cas9 system in the *S. brasiliensis* biological system.

Conclusion

Even without successfully applying the CRISPR/Cas9 system in the *S. brasiliensis* biological system, this technique still has tremendous potential in future studies using different promoters to induce the expression of the CRISPR/Cas9 system. The optimization of this technique can contribute to a better understanding of host-pathogen interaction of *Sporothrix* spp. and help create therapies or strategies against sporotrichosis.

CHAPTER 4 – Set of transformants and plasmids for *Sporothrix brasiliensis*

Abstract

Sporotrichosis is the world's most prevalent and distributed subcutaneous mycosis, and the *S. brasiliensis* species is reported as the most virulent species. However, virulence mechanisms of *Sporothrix* spp. still lack clarification. During the last years, the genome of several strains has been fully sequenced, and the data is available in open access resources; this genomic knowledge has not been accompanied by reverse genetics tools, which could contribute to decoding gene functions. This present chapter focuses on creating a genetic toolbox, allowing the development of a set of transformants that can be used in future studies to help unravel *S. brasiliensis* poorly understood virulence mechanisms. To optimize an ectopic gene expression in *Sporothrix* transformants, we tested two promoters, GAPDH and H2A. Through the ATMT protocol, we produced tagged strains expressing GFP or mCherry in the cytoplasm with the full-length GAPDH promoter, pGAPDH with different bp lengths, and the H2A promoter. Our results show that the expression promoted by the GAPDH promoter led to a high genetic and constitutive expression of the *S. brasiliensis* transformants. Thus, we developed plasmids with the GAPDH or H2A promoter, fluorescent proteins (GFP and mCherry), and a linker sequence between them, allowing the insertion of any localization signal to mediate the transport of the fluorescent proteins from the cytoplasm to the target region. Thus, nuclear gene localization was inserted between the pGAPDH promoter and fluorescent proteins (GFP or mCherry). After sequencing, several of these plasmids were used for the ATMT technique and were successfully used to produce several *S. brasiliensis* fluorescent tag strains. After microscopic analysis, strains exhibited a bright fluorescent signal accordingly to their localization signal (nucleus or cytoplasm). Together, our results confirm the easy and adaptable protocol's success in constructing a set of *S. brasiliensis* strains.

Introduction

Sporotrichosis is the world's most prevalent and dispersed subcutaneous mycosis (5). This endemic disease is caused by the dimorphic fungus from the Sporothrix genus, which is prevalent worldwide in tropical and subtropical areas (2). *Sporothrix brasiliensis* is reported as the most virulent species among those causing sporotrichosis (7,8), but the mechanisms behind this phenomenon still require enlightenment (8). Since the discovery of fluorescence proteins, they have become a powerful tool for analyzing gene expression and protein localization in various experimental systems (90). Consequently, their use can help better understand the pathophysiology of several pathogens, including *S. brasiliensis.*

The GFP and mCherry proteins emit a bright green and red fluorescence, respectively, easily detected by various instrument platforms. They essentially provide a 'fluorescent tag' on a desired protein or structure, making it possible to probe its location, activity, or interaction with other molecules (80,89). Although *S. schenckii* strains expressing fluorescent proteins have already been produced (49), the use of these tools in S. brasiliensis is currently absent.

Transport of all proteins in a eukaryotic cell begins in the cytosol, but organelle proteins must be selectively and efficiently transported into their destination compartments to exercise their physiological functions (91). Nuclear and mitochondrial localization signals (NLS and MLS, respectively) are generally short peptide chains acting as signal fragments, mediating their transport from the cytoplasm into the targeted region (91,92). These localization signals can be merged with fluorescent proteins to conduct them into the desired location inside the cell, essentially allowing the creation of mutants with localized fluorescence (91,92).

Material and Methods

Microorganisms and culture media

The *S. brasiliensis* ATCC MYA-4823 strain (55,100) was used throughout this study. Cells were cultured at 37°C for 72h in yeast extract peptone dextrose (YPD) medium (pH = 7.8). Yeast cells were obtained through sterile gaze filtration to remove cell aggregates and hyphae. The *A. tumefaciens* AGL-1 (101,102) strain harbored each plasmid used in the experiments. *A. tumefaciens* cells were cultured at 26°C in L.C. BROTH medium, supplemented with the antibiotic rifampicin (20µg/mL, Sigma-Aldrich©). To selective medium used kanamycin (100µg/mL, Formedium™) to maintain the plasmids used in this work. The L.B. medium supplemented or not with kanamycin (50µg/mL) was used for *E. coli* growth at 37°C. SOC medium to allow the *E. coli* recovery in the *in vivo* assembly technique (103). For every solid medium, 1.5% of agar was added. Cultures were maintained at 4°C for short-term storage and were regularly sub-cultured every 4–6 weeks.

Primers and Plasmid construction

Primers, plasmids, and inserts were designed using Primer3Web® (https://primer3.org/), and SnapGene® (https://www.snapgene.com/), and all the primers used are shown in Table 4. As a strategy to express fusions of GFP or mCherry with a variety of targeting sequences, plasmids were designed with a specific digestion enzyme site, cut by the AvrII enzyme (New England Biolabs®), and a linker sequence

that allowed us to edit and insert a specific target sequence that directs fluorescence to the desired region in the mutant. For this, we made primers for the H2A, TIM16, and TOM6 sequences. The fragments were generated by overlapping high-fidelity polymerase PCR and inserted into the vector backbone, pPTS608-Cas9-HygB (70), by *in vivo* assembly technique (103,109) and confirmed by Sanger DNA sequencing. Different enzymes were used to linearize the plasmid and allowed your edition. Plasmid DNA extraction, recombinant DNA manipulation, and *E. coli* and *A. tumefaciens* transformation procedures were performed as reported elsewhere (104,107).

Table 4. Primers used to create the fluorescent tag *S. brasiliensis* strains.

Primer name	SEQUENCE	Observations
HPH_F	ATGCCTGAACTCACCGCGAC	HPH resistance gene in PTS608 plasmids and derivatives
HPH_R	TTCTACACAGCCATCGGTCC	
cas9_mcherry4	CAGAACTCTGTCCAGGTCATGTAATATCACACGCAGCCGAAGCCTTCGAG	Plasmid construction: Cas9-mCherry
H2A-mcherry1	ATGGTGAGCAAGGGCGAGGA	Plasmid construction: pH2A-mCherry and pH2A-H2A-mCherry
H2A-mcherry2	CCATGTTATCCTCCTCGCCC	
H2A-mcherry3	ATGGCTGGCGGCAAGGGAAAAT	
H2A-mcherry4	ATCTGCGGAACATATACTGGGCCCGGGAATTCCGGTGACGTTTGGTGCAG	
H2A-mcherry5	CCGACGATTTTCCCTTGCCGCCAGCCATTGTGTCACGAAAGCGAAGTTG	
H2A-mcherry6	ATGTTATCCTCCTCGCCCTTGCTCACCATTGTGTCACGAAAGCGAAGTTG	
GAPDH_promoter_1	CATCTGCGGAACATATACTGGGCCCGGGAACTCGCTCGTCTTGGAGTGCT	Plasmid construction: promoter GAPDH-mCherry or GFP
GAPDH_promoter_2	CTCCTCGCCCTTGCTCACCATTTTGACTTTGGAATTGAGTTAGCC	
seq_pH2A	GCCTTACTGTATCGCCTCTG	sequencing primers
seq_pGADPH	CCTGGTGACGACTGGCTC	sequencing primers
Linker_GFP	AGTTCTTCTCCTTTACTCATACCAGAGGTGGCGACCGGTG	Plasmid construction: GFP gene and H2A location sequence
H2A_GAPDH	ATTTTCCCTTGCCGCCAGCCATTTTGACTTTGGAATTGAGTTAGCC	
GFP_F	ATGAGTAAAGGAGAAGAACTTTTCAC	
pGAPDH_GFP	AGTTCTTCTCCTTTACTCATTTTGACTTTGGAATTGAGTTAGCC	Plasmid construction: promoter GAPDH with different sizes
GAPDH_1000	GAACATATACTGGGCCCGGGAAGCAGCCCATGACGACCCATT	
GAPDH_800	GAACATATACTGGGCCCGGGAATGCTACCCCGCCATCGATCG	
GAPDH_c/UTR_R	GATCCGTGCTTCCCGATGCCCCTAGGTTGACTTTGGAATTGAGTTAGC	strategy with pGAPDH and linker
GAPDH_s/UTR_R	GATCCGTGCTTCCCGATGCCCCTAGGGAAACTCGAACCGGAG	
F_Tim16_GFP	AACTCAATTCCAAAGTCAACATGGTACGTACACGCAAG	TIM16 localization gene sequence
R_Tim16_GFP	ATCCGTGCTTCCCGATGCCCTCCGGTCCTTGTACATCTTGGGCTTG	
F_Tom6_GFP	AACTCAATTCCAAAGTCAACATGTACCAGCCAAGACAGATCG	TOM6 localization gene sequence
R_Tom6_GFP	ATCCGTGCTTCCCGATGCCCTCGGCGGCAGCAGGA	

Plasmids pH2A-mCherry and pGAPDH-mCherry

The pPTS608-Cas9-HygB backbone vector was cut with the enzymes BglII (New England Biolabs®) and PmlI (New England Biolabs®), and two fragments were inserted. For the plasmid with pH2A-mCherry – The first used primers H2A-mcherry4 and H2A-mcherry6 to amplify the H2A promoter from the gDNA of *S. brasiliensis*. The second used primers cas9_mcherry4 and H2A- mcherry1 to amplify the mCherry. For the plasmid with pGAPDH-mCherry, we used GAPDH_promoter_1 and GAPDH_promoter_2 primers to amplify the GAPDH promoter from the *S. brasiliensis* gDNA, and the primers cas9_mcherry 4 and H2A-mcherry1 to amplify the mCherry.

Plasmids pH2A_H2A-mCherry and pGAPDH_H2A-GFP

The pPTS608-Cas9-HygB backbone vector was cut with the enzymes BglII (New England Biolabs®) and PmlI (New England Biolabs®), and three fragments were inserted.

pH2A_H2A-mCherry – (1) used primers H2A-mcherry4 and H2A-mcherry5 to amplify the H2A promoter from *S. brasiliensis* gDNA. (2) used primers H2A-mcherry2 and H2A-mcherry3, the coding region of the H2A gene, a linker sequence, and the initial part of the mCherry fluorescence protein gene. (3) Primer cas9_mcherry 4 and H2A-mcherry1 were used to amplify the mCherry.

pGAPDH_H2A-GFP – (1) GAPDH promoter amplified from gDNA *S. brasiliensis* (primers GAPDH_promoter_1 and H2A_GAPDH). (2) target sequence H2A (primers H2A-mcherry3 and Linker_GFP). (3) GFP gene (primers cas9_mcherry 4 and GFP_F).

Plasmids GFP and Promoter GAPDH with different sizes

The plasmids were designed: pPTS608_pGAPDH-GFP, pPTS608_p1157GAPDH-GFP, pPTS608_p757GAPDH-GFP, and pPTS608_pGAPDH-without_5'UTR-GFP. The pPTS608-Cas9-HygB plasmid was digested with the enzymes BglII and PmlI, and two fragments were inserted. The first used gDNA from *S. brasiliensis* to amplify the GAPDH promoter, and promoter primers vary depending on the size of the desired promoter. For the complete GAPDH promoter primers GAPDH_promoter_1 and pGAPDH_GFP, the 1157 bp primers GAPDH_1000 and pGAPDH_GFP, and the 757 bp promoter primers GAPDH_800 and pGAPDH_GFP. The second remains the same across all plasmids and is designed to amplify the GFP gene with primers cas9_mcherry4 and GFP_F. For the construction of pPTS608_pGAPDH-without_5'UTR-GFP, a different strategy was used; the Plasmid pPST608_GAPDHfull_H2A_GFP was used as a vector backbone and digested with ApaI and XhoI and

the promoter to be inserted made by amplifying the *S. brasiliensis* genomic gDNA with the primers GAPDH_promoter_1 and GAPDH_s/UTR_R. Plasmid maps are shown in Figure 14.

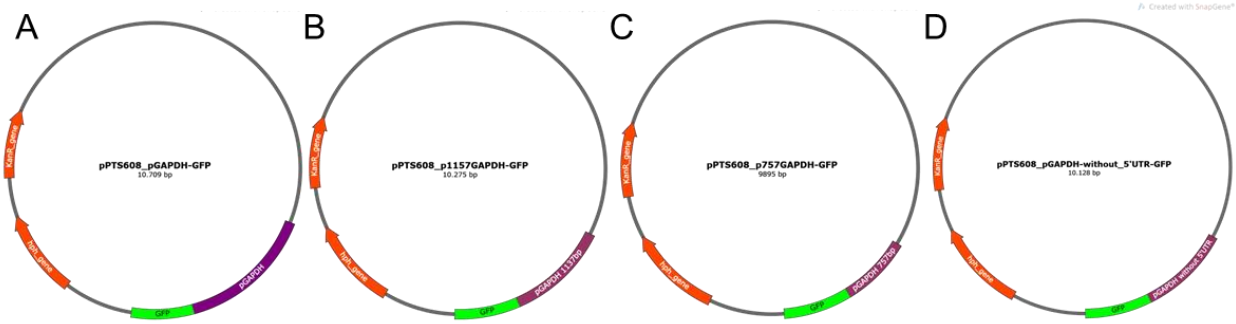


Figure 14. Plasmid PTS608 with different versions of the GAPDH promoter. (A) pPTS608_pGAPDH-GFP, exhibiting the full-length pGAPDH. (B) pPTS608_p1157GAPDH-GFP, with a 1157bp of the pGAPDH. (C) pPTS608_p757GAPDH-GFP, with only 757bp of the promoter's sequence. (D) pPTS608_pGAPDH-without_5'UTR-GFP, depicting the pGAPDH without the 5'UTR region.

pPTS608_pGAPDH-linker-GFP and pPTS608_pGAPDH-linker-mCherry

For the design of plasmids that express fusions of fluorescence proteins with a specific region of the fungal cell, new plasmids with the fluorescence genes GFP and mCherry were produced. The plasmids pPTS608_pH2A_H2A-mCherry and pPTS608_pGAPDH_H2A-GFP were used as a backbone vector. They were digested with ApaI (Thermo Fisher Scientific) and XhoI (Thermo Fisher Scientific) enzymes. After digestion, an amplified fragment of *S. brasiliensis* gDNA containing the desired promoter is inserted. These plasmids retain the original vector linker in addition to receiving the AvrII cleavage site, allowing easy editing and insertion of the desired location sequence. Its backbone vector is pPTS608_pGAPDH_H2A-GFP or pPTS608_pH2A_H2A-mCherry, which is digested as above. These are plasmids without localization sequences containing linker, GFP or mCherry gene sequence, and AvrII enzyme digestion site. Its promoter is amplified using primers GAPDH_promoter_1 and GAPDH_c/UTR_R in *S. brasiliensis* gDNA.

pPTS608_pGAPDH-TIM16-GFP and pPTS608_pGAPDH-TIM16-mCherry

Backbone plasmids depend on the desired fluorescence gene, which can be pPTS608_pGAPDH-linker-GFP and pPTS608_pGAPDH-linker-mCherry. After linearizing with the AvrII enzyme, a fragment containing the TIM16 gene localization sequence is inserted. The TIM16 sequence fragment is amplified from *S. brasiliensis* gDNA and uses primers F_Tim16_GFP and R_Tim16_GFP (Figure 15).

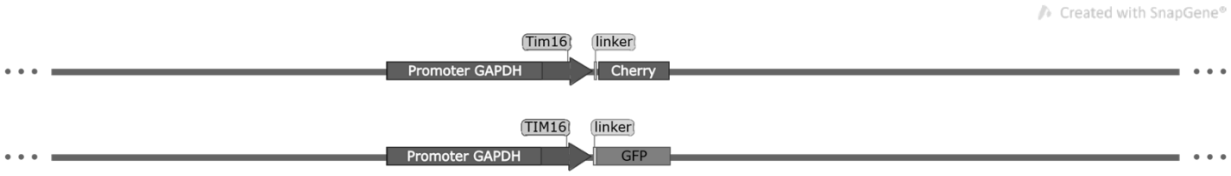


Figure 15. Plasmid with the TIM16 target sequence. Plasmids with promoter GAPDH, TIM16 target sequence in fusion with fluorescent protein mCherry or GFP.

pPTS608_pGAPDH-TOM6-GFP

This plasmid used the same strategy as in the plasmids containing the TIM16 sequence. However, in this case, the amplified sequence of the genome of *S. brasiliensis* is the TOM6 target sequence using primers F_Tom6_GFP and R_Tom6_GFP (Figure 16).

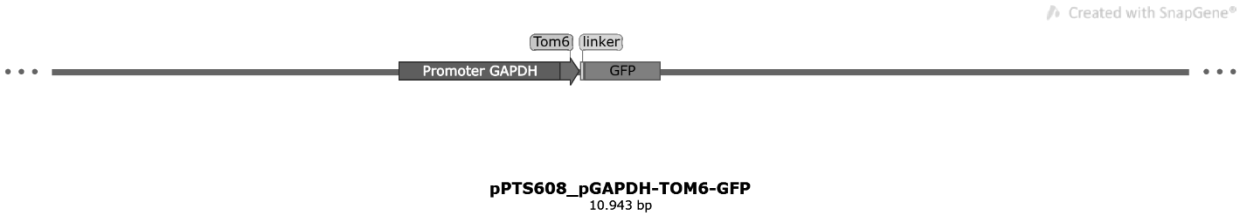


Figure 16. Plasmid pPTS608_pGAPDH-TOM6-GFP. Plasmid with promoter GAPDH and TOM6 target sequence in fusion with GFP.

In vivo assembly protocol

The adapted in vivo assembly technique was used to construct the plasmids. The vector was linearized with the desired enzymes, and inserts were made using Invitrogen™ Platinum™ SuperFi™ DNA Polymerase. A ratio of 1:5 (linearized plasmid:each insert) was used (103), with 1 equal to 20 ng of DNA. After calculation, the values were placed in a 25 µL aliquot of NZYStar® competent cells. The transformation protocol was followed according to the company's recommendations; however, for the cell's incubation, 250µL were used, and their total volume was plated in a selective medium. The schematization of the *in vivo* assembly protocol can be seen in Figure 17.

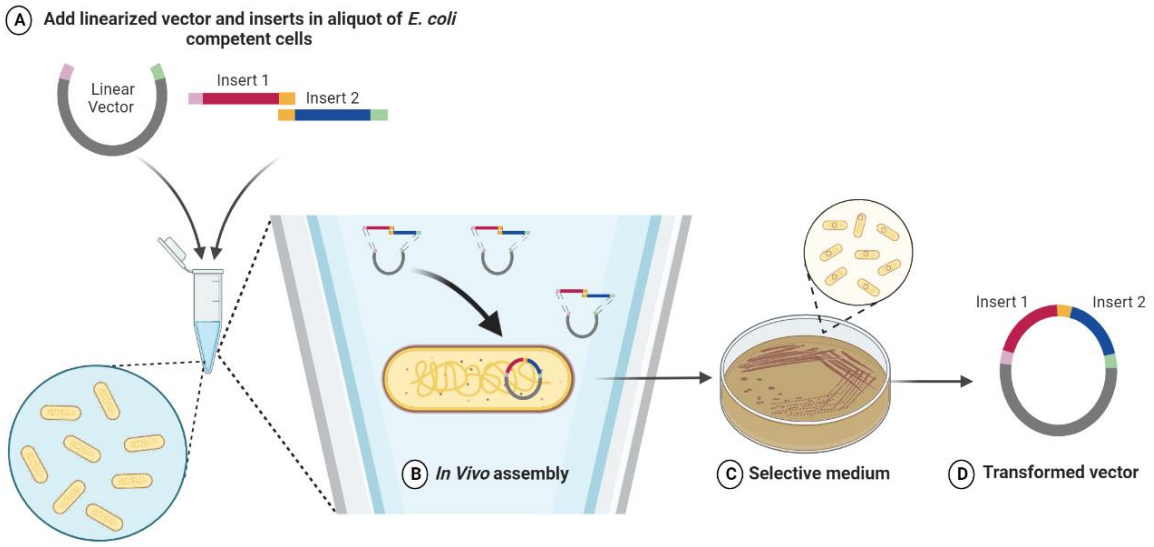


Figure 17. The schematization of the *in vivo* assembly protocol. (A) Linearized vectors and inserts, after calculated, are added to an aliquot of *E. coli* competent cells. (B) Upon the realization of the transformation protocol, the inserts and the linearized plasmid enter the bacterial cells, whose own machinery is responsible for constructing the plasmids. (C) Only cells that successfully produce the desired plasmid grow when plated in a selective medium. (D) Bacteria containing the desired vector are grown, and the plasmid is extracted for use in further experiments.

Fluorescent microscopy

S. brasiliensis transformants were produced through ATMT with the AGL-1 strain harboring several plasmids, which confers the expression of the fluorescence proteins GFP or mCherry in the cytoplasm, nucleus, or mitochondria. Yeast and mycelium cells of ATCC MYA-4823 transformants and wild type were obtained during exponential growth by incubation at 37 and 26°C, respectively, for 24h while shaking 200rpm. Cells were fixed with 500µL of 2% paraformaldehyde for 30 minutes, R.T., while shacking and stored at 4°C until analysis. Fluorescence in the nucleus was confirmed by co-localization with Invitrogen™ DAPI (4[prime],6-diamidino-2-phenylindole). Exponential growth cells were stained with 0.1µg/mL of DAPI for an hour at R.T. while shaking. To allow better mitochondrial visualization. Fluorescence microscopy analyses were performed with the Olympus Widefield Upright Microscope BX61 by either bright-field or fluorescent microscopy. All images were captured using 395nm/509nm for excitation and emission, respectively, and exposition time to the laser beam was automatically set. Imagens were treated in the ImageJ© (https://imagej.nih.gov/ij/) software.

Promoter evaluation

To analyze the MFI difference between the GAPDH and H2A promoters, six mutants of each and *S. brasiliensis* wildtype cells were cultured for 3 days at 37°C, 200 rpm in a 100 mL Erlenmeyer flask with 20 mL of YPD (pH 7.8). Mutants grew on a selective medium (150 µg/mL hygromycin B, Formedium™). The OD_{660nm} was periodically measured, and yeast cells were fixed with paraformaldehyde at a concentration of 2% for 15 minutes at 4°C. Thirty thousand events were captured using FACS, and the MFI of singlets of each condition was measured and compared. Wildtype yeast cells in each period were used to determine the autofluorescence threshold. Next, the MFI of mutants with different lengths of the GAPDH promoter were compared: full-length (1571 base pairs), 1137bp, 757bp, 550bp, and pGAPDH without its 5' untranslated region (5'UTR). Through an initial FACS screen, the MFI of 550bp-GAPDH promoter mutants was below or equal to the autofluorescence threshold of wildtype cells (data not shown). Then, five randomly selected transformants with full-length promoters, 1137bp, 75bp, and without the pGAPDH 5'UTR region were cultured in a YPD selective medium (pH 7.8). OD_{660nm} was set to 0.1 and cultured at 37°C for 24h to achieve exponential growth. Yeast cells were fixed with 2% paraformaldehyde for 15 minutes at 4°C, and thirty thousand events were captured using FACS. The singlet MFI of each condition was measured and compared. Wildtype yeast cells at each period were used to determine the autofluorescence threshold.

Statistical analysis

The promoters' evaluation data were obtained on a BD FACS LSRII instrument (B.D. Biosciences) and processed using FlowJo© v.10 software (Tree Star Inc., https://www.flowjo.com/). Data are reported as the mean ± standard error of the mean (SEM) of at least two independent assays with two or three replicates. The statistical analysis was performed using GraphPad Prism Software version 8.0 (GraphPad Software Inc, California, USA, https://www.graphpad.com/scientific-software/prism/). The normality assumptions were assessed in all cases using the Shapiro-Wilk test. The MFI differences between promoters in different periods were evaluated using Two-Way ANOVA. Statistically significant values are indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

Results and Discussion

Fluorescent tag *S. brasiliensis* strains

To optimize an ectopic gene expression in *Sporothrix* mutants, we evaluated two promoters from *S. brasiliensis*, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and histone 2A (H2A). Both of these genes are known in other systems to be driven by strong promoters where pGAPDH is constitutive, and pH2A expression is associated with the growth phase. GAPDH is a catalytic enzyme involved in glycolysis (110), and at least one copy of this highly expressed gene is present in several filamentous and thermally dimorphic fungi (111,112). The GAPDH promoter in yeast, TDH3, exhibits high transcriptional activity (113–115). H2A is a histone protein essential for chromatin organization, and its promoter has already been used to drive gene expression in other systems (116–118). Through the ATMT protocol, we produced tagged strains expressing green fluorescence protein (GFP) or red fluorescence protein (mCherry) with the promoter GAPDH and expressing red fluorescence protein (mCherry) with the promoter H2A that is stable both at yeast and mycelium forms (Figure 18). Moreover, similar to other organisms, our data show that these proteins are localized in the cytosol when expressed without any localization target.

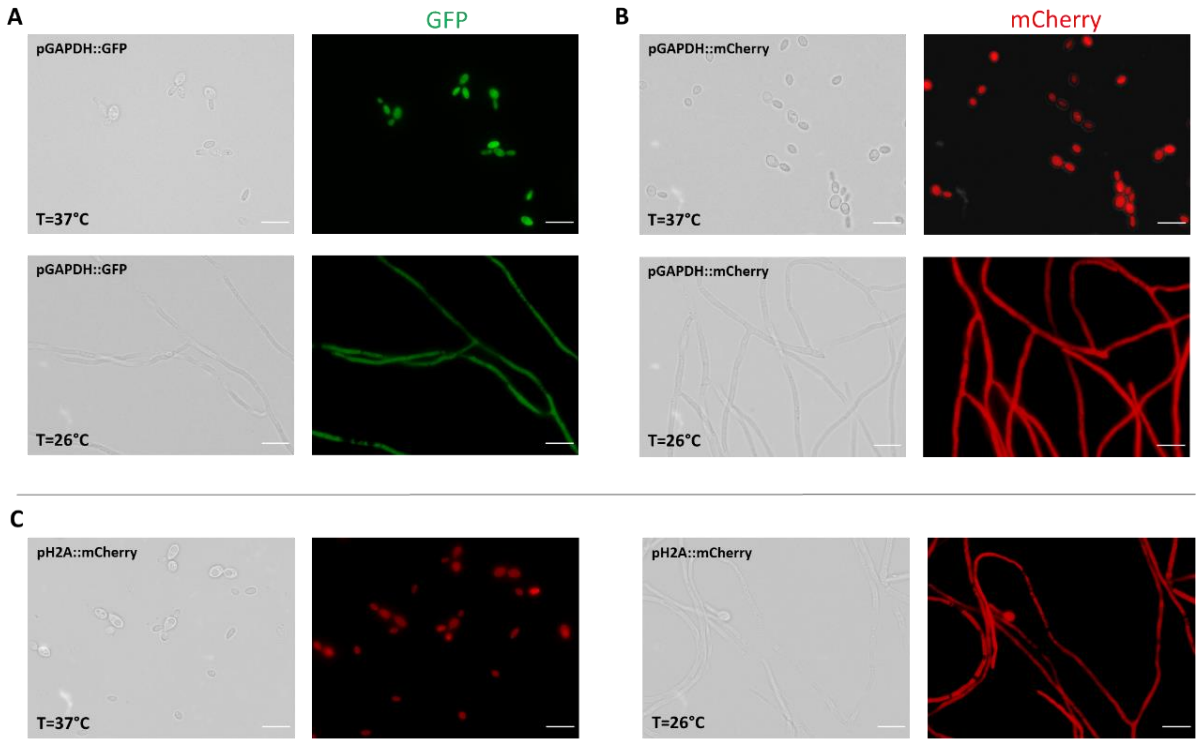


Figure 18. Analysis of different *S. brasiliensis* transformant promotors through GFP and mCherry gene expression. (A) Fluorescence microscopic analysis of randomly selected *S. brasiliensis* transformant expressing GFP (promoter GAPDH) during both yeast and mycelium phases, through Bright

field and FITC channels. The scale bar equals 10µm for all images. (B) Fluorescence microscopic analysis of randomly selected *S. brasiliensis* transformants expressing mCherry (promoter GAPDH) during both yeast and mycelium phases, through Bright field and TRITC channels. The scale bar equals 10µm for all images. (C) Fluorescence microscopic analysis of randomly selected *S. brasiliensis* transformants expressing mCherry (promoter H2A) during both yeast and mycelium phases, through Bright field and TRITC channels. The scale bar equals 10µm for all images.

Promoters' comparison in *S. brasiliensis* gene expression

Then, to further characterize the ectopic gene expression in *Sporothrix* transformants, we compared by flow cytometry the mean fluorescence intensity (MFI) of the red fluorescence driven by either pGAPDH or pH2A during batch growth in glucose medium. Our data show that the pGAPDH promoter had a higher MFI during all phases of growth when compared with that obtained for pH2A ($p \leq 0.0001$, Figure 19). Furthermore, no difference in red fluorescence was detected throughout both exponential and stationary growth phases with the pGAPDH promoter, while the MFI of the pH2A decreased at the later stage of the exponential growth (50h of incubation; $p \leq 0.0126$, Figure 19).

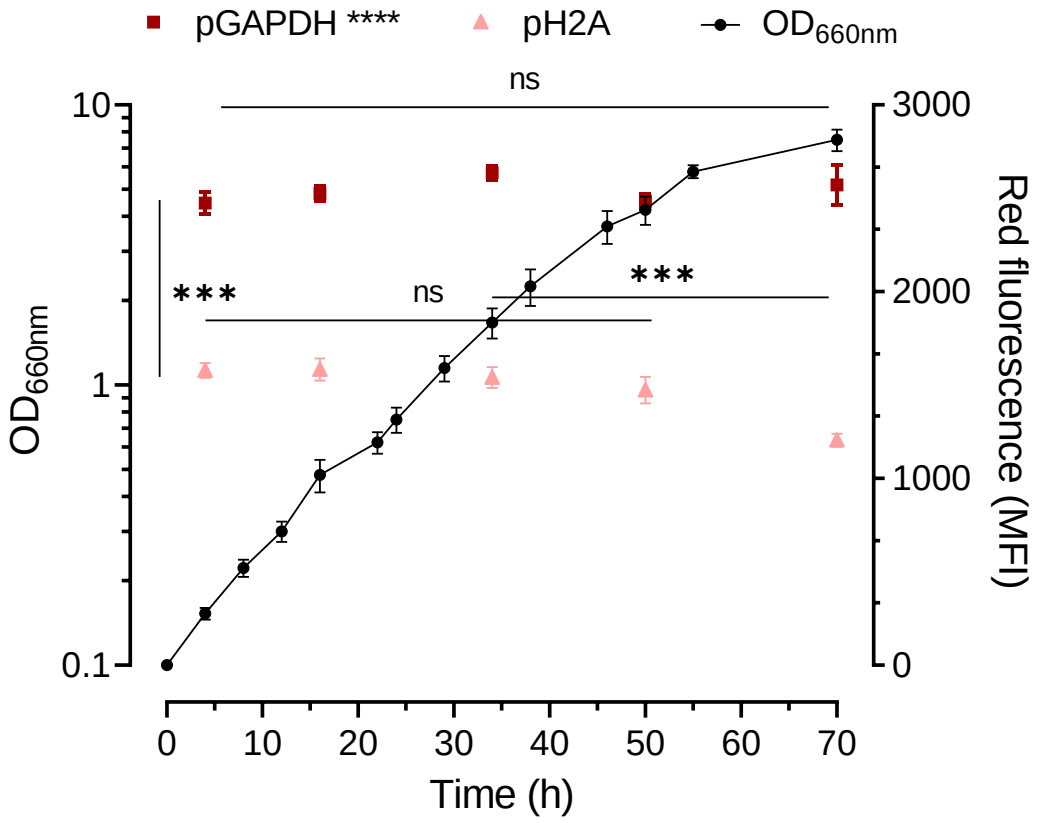


Figure 19. Differences between pGAPDH and pH2A promoters in *S. brasiliensis* mCherry expression. Data depicts the OD_{660nm} ± SEM, illustrated as dots, correlated with the mean of MFI

expressed by pGAPDH and pH2A promoters through time (n=5), demonstrated as squares and triangles, respectively. The pGAPDH promoter had a higher MFI than the pH2A promoter at all time points (p<0.0001). Two-Way ANOVA determined statistically significant data by Sidak's multiple comparisons test****(p<0.0001), *(p<0.5).

Gene expression evaluation in a different version of the GAPDH promoter in *S. brasiliensis*

For cloning plasmids their size can affect their gene transfer efficiency (119) as well as are more likely to contain potentially unstable regions (120). Therefore, in molecular biology the reduction of all the plasmid features to the smallest lenth regions are essential. Promoters are known to have, in general, three regions: the core, upstream activating sequence (UAS), and 5' untranslated region (5'UTR) (120). The core promoter region in eukaryotes is located most proximal to the start codon and contains the RNA polymerase binding site, TATA box (121), while other areas can be responsible for regulatory and transcription roles. Consequently, we compared GFP expression using different sizes of the GAPDH promoter. Knowing that this promoter drives gene expression at a constitutive level throughout the distinct phases of the growth curve, we compared MFI for all constructs only at the exponential growth phase. As shown in Figure 20, only the full-length pGAPDH mutants had a significantly higher MFI than wildtype cell autofluorescence (p<0.0001). The 5'UTR is a DNA regulatory region located at the 5' end of all protein-coding genes transcribed into mRNA. It contains several regulatory components that may be essential in controlling translation (120). Removal of this region severely affected GFP expression (Figure 20, p<0.0001). Together, these results support the conclusion that the full-length pGAPDH we selected initially drives the strongest expression level in *S. brasiliensis* transformants.

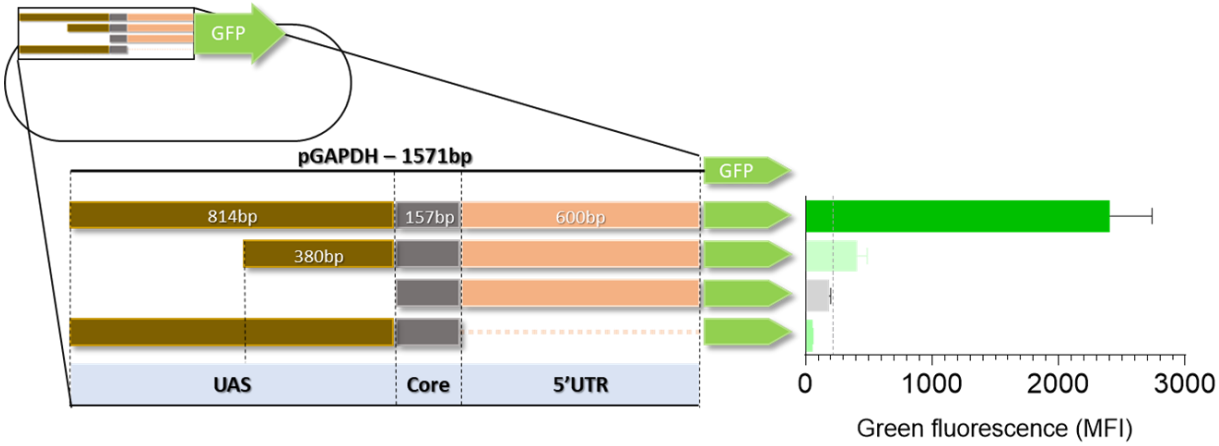


Figure 20. GFP expression differences between GAPDH promoter size. Full-length, 1100bp, 800bp, and pGAPDH without 5'UTR in *S. brasiliensis.* Bars depict the mean ± SEM number of MFI per condition (n=5).

Fluorescent proteins fusion constructions in *S. brasiliensis*

To create several fluorescent tag strains from the *S. brasiliensis* ATCC MYA-4823 strain, plasmids were developed with the pGAPDH promoter, fluorescent proteins (GFP or mCherry), and a linker sequence (Figure 21).

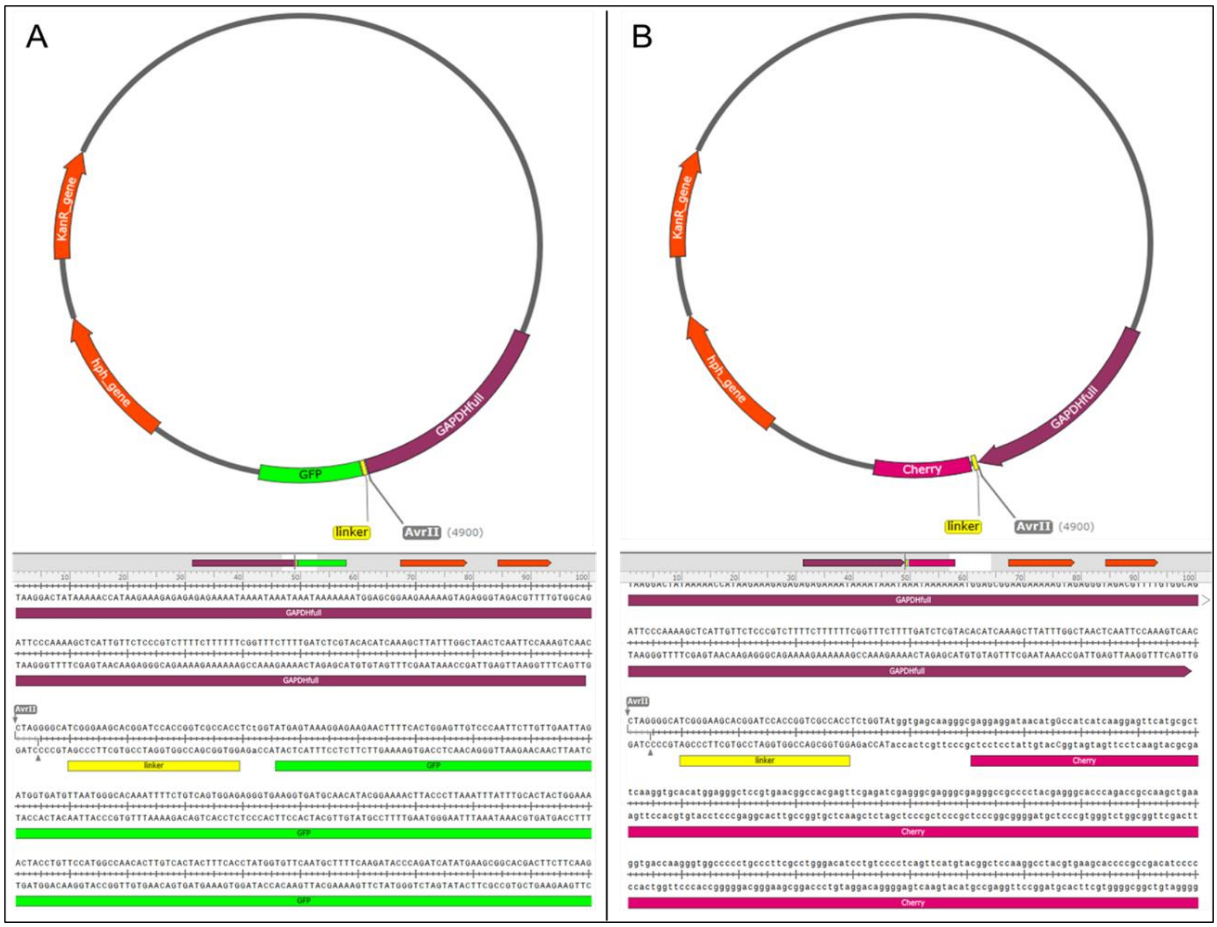


Figure 21. Plasmids schematization with the GAPDH promoter, cleavage site (AvrII), linker, and fluorescence protein. (A) Plasmid with promoter GAPDH, AvrII digestion site, linker sequence, and GFP. (B) Plasmid with promoter GAPDH, AvrII digestion site, linker sequence, and mCherry.

The linker sequences are known to contribute to achieving stable conformations in chimeric fusion proteins, allowing the construction of in-frame protein fusion. In this line, with the plasmids, we could insert any localization signal, short peptide sequences that act as signal fragments, mediating their transport from the cytoplasm into the targeted region (91,92), or any protein. To evaluate its localization as proof of principle, we chose the H2A gene (gene encoding nuclear proteins) that has already been successfully used in another fungus (122). We cloned it between the pGAPDH promoter and fluorescent proteins creating the plasmids pGAPDH::H2A::GFP and pH2A::H2A::mCherry, leading to

N-terminal in-frame fusions. After sequencing, these plasmids were used in ATMT transformations to successfully produce the nucleus fluorescent tag strains from the *S. brasiliensis*.

Upon microscopic analysis, the positive transformants, resistant to hygromycin, exhibited a bright fluorescent signal. The co-localization of the DAPI stain demonstrated that both pGAPDH::H2A::GFP and *S. brasiliensis* pH2A::H2A::mCherry transformants expressed the fluorescence protein in the nucleus (Figure 22).

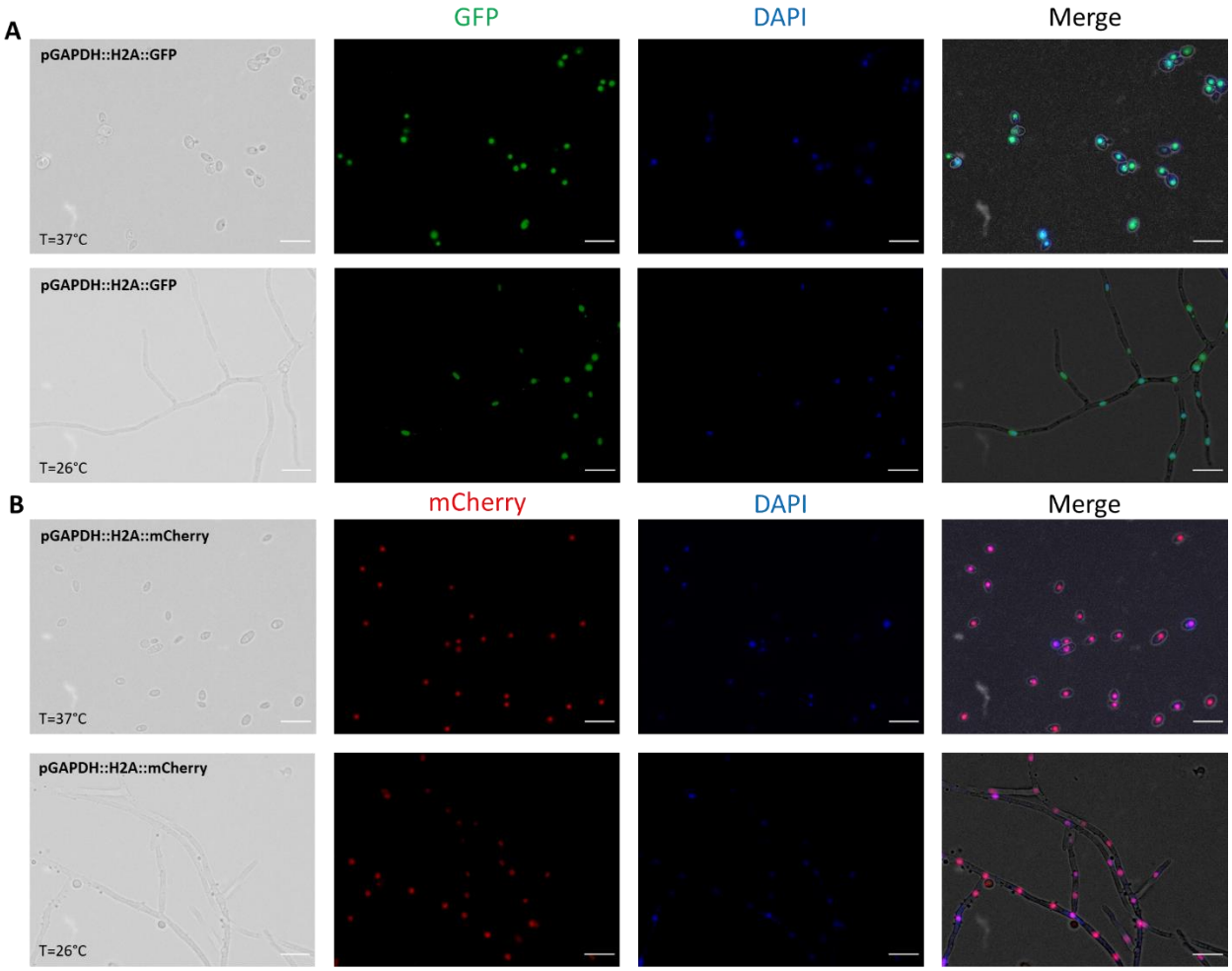


Figure 22. *S. brasiliensis* tag strains expressing fluorescent proteins in the nucleus. The scale bar equals 10μm for all images. The merged images show high co-localization between GFP and mCherry expressions with the DAPI (4[prime],6-diamidino-2-phenylindole) nuclei staining. (A) Fluorescence microscopic analysis of pGAPDH::H2A::GFP isolates, expressing GFP in the nucleus during yeast and mycelium phases. (B) Fluorescence microscopic analysis of pH2A::H2A::mCherry isolate, expressing mCherry in the nucleus during both yeast and mycelium phases. The merged images show high co-localization between GFP and mCherry expressions with the DAPI (4[prime],6-diamidino-2-phenylindole) nuclei staining.

For the mitochondrial localization genes, we chose following the same way as we decided on the H2A gene. The TIM16 or Pam16 is a subunit of the translocase of the inner mitochondrial membrane, which localizes mainly to the internal boundary membrane. TOM6 is a component of the TOM (translocase of outer membrane) receptor complex responsible for recognizing and translocating cytosolically synthesized mitochondrial preproteins. They are genes previously used to produce tagged strains transformants (123–125). The plasmids with pGAPDH::TIM16::mCherry, pGAPDH::TIM16::GFP, and pGAPDH::TOM6::GFP were successfully produced as the previous ones, but it was not yet possible to carry out the ATMT protocol to verify if the chosen strategy will be successful in carrying out the mitochondrial localization in the transformants.

Conclusion

The GAPDH constitutive promoter exhibited a higher gene expression and is a promising candidate for stablishing the CRISPR/Cas9 system in *S. brasiliensis.* This promoter together with AvrII restriction enzyme were used to create several *S. brasiliensis* fluorescent strains, expressing the fluorescent proteins GFP or mCherry, either attached or not to localization genes, which guided these fluorescent proteins into specific organelles, such as the nucleus. Taken together, our results confirm the success of easy-to-edit protocol to build a set of *S. brasiliensis* transformants using ATMT and plasmid construction techniques. These techniques allow us to create a set of mutants to tag and analyze the structure, function, and dynamics of *S. brasiliensis* possible virulence factors, thus helping to unravel pathogenicity of this microorganism.

CHAPTER 5 – General discussion and further perspectives

Sporotrichosis is the most prevalent and distributed subcutaneous mycosis globally (5). This disease is endemic and caused by the dimorphic fungus of the genus Sporothrix, within the order Ophiostomatales, generally associated with non-pathogenic environmental fungi strongly related to the decomposition of wood, plants, and soil (15). However, members of this genus are successful thermally dimorphic mammalian pathogens, causative agents of human and animal sporotrichosis (1,3,10,15). The mechanisms responsible for this are still poorly described and need further studies (7,8). *S. brasiliensis* is particularly reported as the most virulent species, exhibiting the worst clinical manifestations (7,8), and driver hyperendemic situations in Brazil. Virulence factors from this species are unknown, and the resistance to antifungal drugs is increasingly being reported (18,126).

The development of genetic editing techniques is essential and valuable for studying and understanding these poorly described mechanisms. For example, the CRISPR/Cas9 gene editing system, which involves the activity of ribozymes to generate the active sgRNA, and when co-expressed with Cas9, the target sequence is modified (70) allowing the creation of mutant with the desired gene edition. The development of the CRISPR/Cas9 techniques in *S. brasiliensis* would be necessary as a gene-editing tool that can help uncover and enlighten underlying mechanisms and was our first aim in Chapter 3. To that, we used the pPTS608-Cas9-Hyg plasmid with the TEF1-αpromoter. Even though the TEF1-αpromoter has already been reported as a suitable promoter to express the CRISPR/Cas9 system in filamentous and dimorphic fungi (67,70), our data show that it is unable to drive the CRISPR/Cas9 gene expression in the *S. brasiliensis* biological system. For this reason, the use of the promoters GAPDH or H2A presented in Chapter 4, may be a possible and quick strategy to be used in the optimization of the CRISPR/Cas9 system in the *S. brasiliensis*.

The Chapter 4 focused on a creation of a genetic toolbox, allowing the development of a set of transformants with fluorescence proteins that can be used in future studies. Through the ATMT protocol, we successfully produced transformants expressing GFP or mCherry in the cytoplasm and nucleus with the GAPDH and H2A promoters. The pGAPDH had a higher gene expression in the transformants. Fluorescent proteins are a powerful tool that enable research experimentation with living cells (82,83). The created *S. brasiliensis* fluorescent tag strains be used in fungicidal and phagocytosis assays. Due to the fluorescence proteins, mitotic stability can be evaluated through flow cytometry, and the transformants can be used for subsequent transformations.

In a short time, using easy-to-edit plasmids, we can test various localization signals, such as those related to mitochondria (whose plasmids have already been produced). And after its optimization,

subsequent oxidative stress studies could be performed, allowing their mitochondria characterization and quantification.

Together, our results confirm the easy and adaptable protocol's success in constructing a set of *S. brasiliensis* transformants through ATMT and plasmid construction techniques. The creation of a genetic toolbox, allowing the development of a set of transformants, can be used in future studies to help unravel *S. brasiliensis'* poorly understood virulence mechanisms. In the future, we aim to use the pGAPDH promoter to express the CRISPR/CAS9 machinery in the *S. brasiliensis* biological system. Using the auxotrophic selection marker 5-FOA and the same sgRNA, we intend to transform and select SPBR_01718- transformants and evaluate the gene deletion through genome sequencing. In fact, our group has recently performed a dual RNA-sequencing on macrophages infected with *S. brasiliensis* and *S. schenckii* aiming to identify potential virulence factors that provide a competitive advantage to *S. brasiliensis*. This approach has revealed a specific transcriptome signature of *S. brasiliensis* absent in *S. schenckii*, where sialidase-1 was identified on the top list of the up-regulated genes (upregulation of 700-fold). This work lay the foundations for the creation and characterization of transformants that overexpress or silence sialidase-1 through an optimized CRISPR/CAS9 system might help explain *S. brasiliensis* increased virulence.

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