Codon optimization and promoter selection facilitates use of *Mucor circinelloides* for reporter assays to monitor infection and antifungal efficacy

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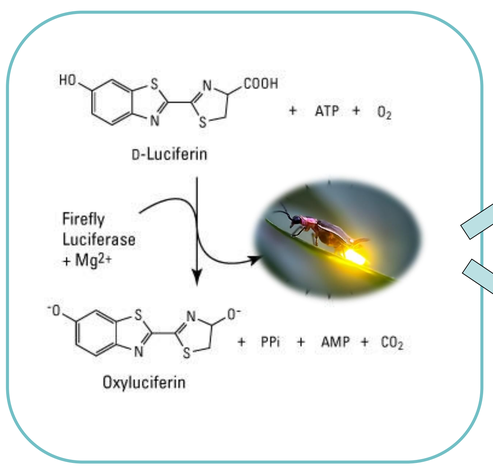
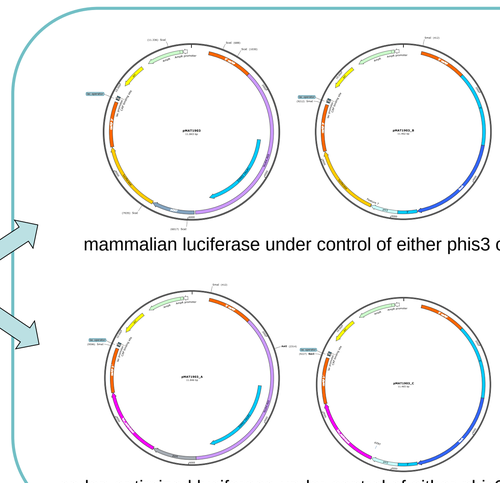
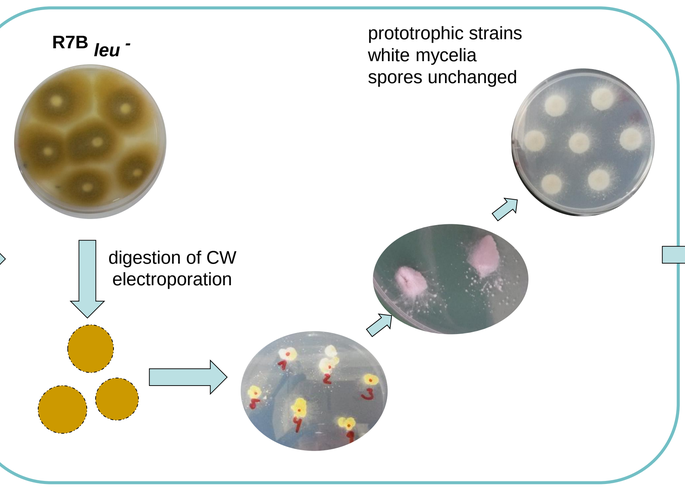
Purpose

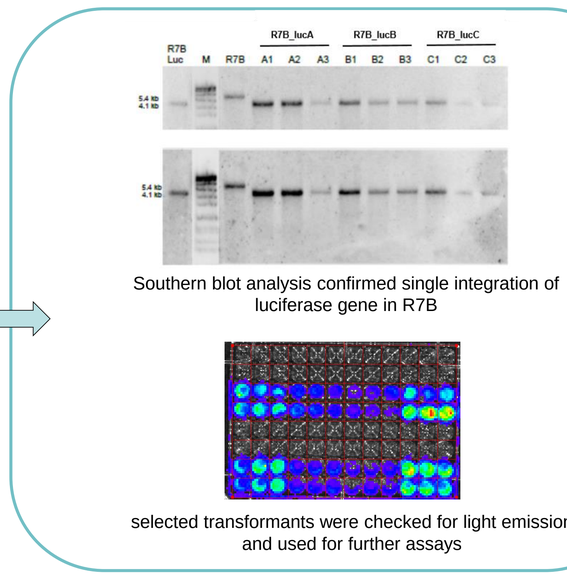
Invasive infections caused by mucormycetes are increasingly seen in the clinics and are still associated with unacceptable high mortality rates. In the Covid pandemic these infections, mainly the rhinocerebral form, are being reported at alarming frequency in India and other countries. Still, little is known about the biology of the pathogens, the establishment and progression of the infection, antifungal resistance mechanisms and successful therapy. Therefore, we aimed to generate tools for (1) alternative methods of drug testing *in vitro*, (2) non-invasive monitoring of the infection in *Galleria mellonella*, (3) visualization of antifungal efficacy and (4) reporter based gene transcription analysis.

Generation of *M. circinelloides* reporter strains









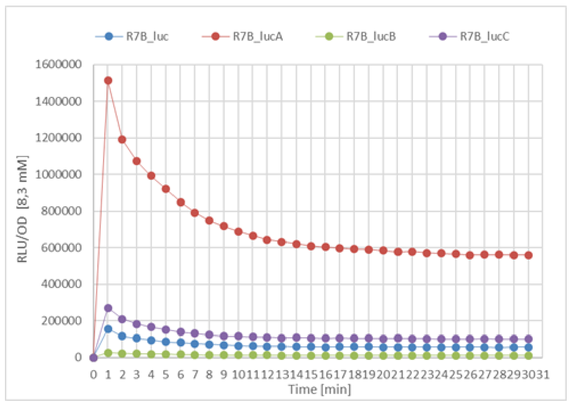
Southern blot analysis confirmed single integration of luciferase gene in R7B

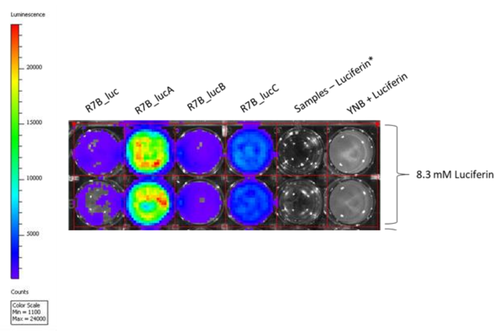
selected transformants were checked for light emission and used for further assays

Firefly luciferase – mammalian or codon optimized (without the sequence for peroxysomal localisation) was cloned in a plasmid containing functional *leuA* gene and either the constitutive promoter of the *M. circinelloides zrt1* gene, or the *his3* gene. Linear plasmid was used to transfect *M. circinelloides* protoplasts of leucine auxotrophic strains. Identification of positive transformants was facilitated by targeted integration of the construct in the *carRP* gene, resulting in the formation of white colonies when integration occured. Homokaryotic transformants were checked for gene integration by PCR and southern blot confirmed single integration of the luciferase gene. Light emission was measured under various conditions and normalized to biomass (OD). Selected strains were used to determine antifungal susceptibility, virulence potential and *in vivo* monitoring of mucormycosis in *Galleria mellonella*.

Functional analysis of luciferase containing *M. circinelloides* strains

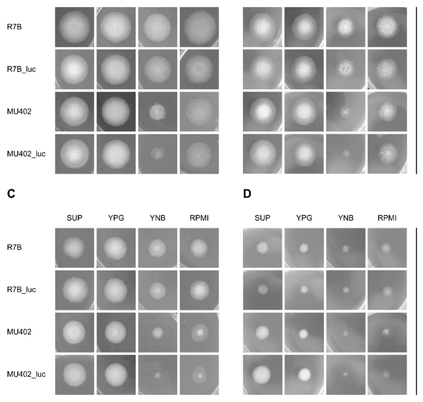
measurement and visualization of light emission

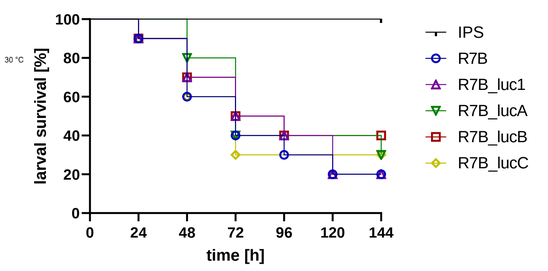




- firefly luciferase is expressed in *M. circinelloides* & light emission can be detected (luminometer) and visualized (IVIS system)
- codon optimization increased light emission, which was normalized to optical density, by 10 time under p*zrt* control (R7B_lucA), and 2 x under control of p*his3* (R7B_lucC) compared to non-optimized luciferase
- light emission decreased with time, but was still detectable after 30 min

growth, virulence potential & antifungal susceptibility profile

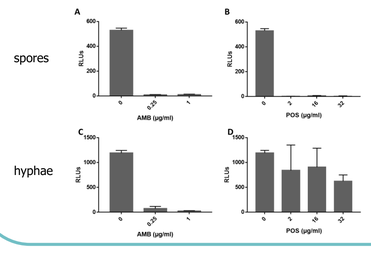




	MIC (µg/ml)	
	PCZ	AMB
R7B	> 8	2
R7B_lucA	> 8	2
R7B_lucB	➢ 16	2
R7B_lucC	> 16	2

- growth, virulence potential in *Galleria mellonella* and antifungal susceptibility pattern to posaconazole and amphotericinB is not altered by the insertion of the luciferase gene

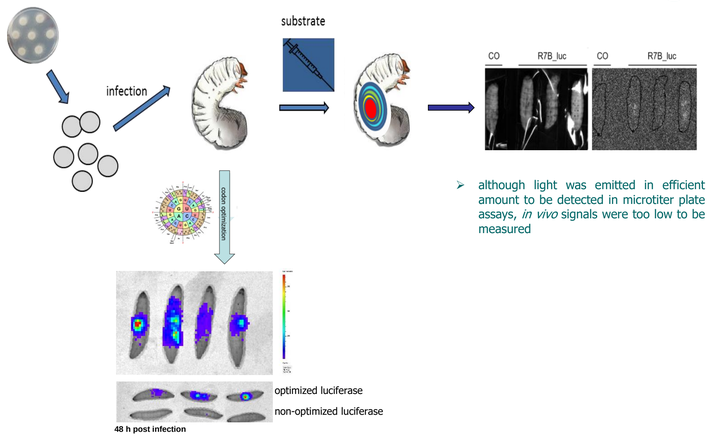
graphical analysis of drug efficacy by detection of light emission



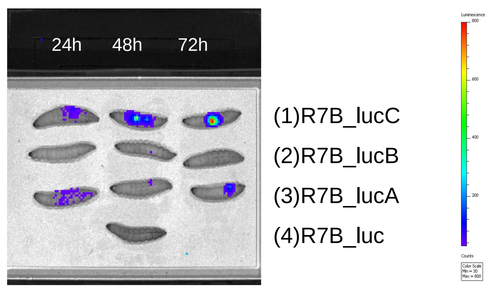
- lack of light emission correlates with growth inhibition, hyphal damage and fungistatic/fungicidial activity
- reporter strain is suitable to evaluate efficacy of antifungal drugs against spores and hyphae

Binder et al. Genes 2018

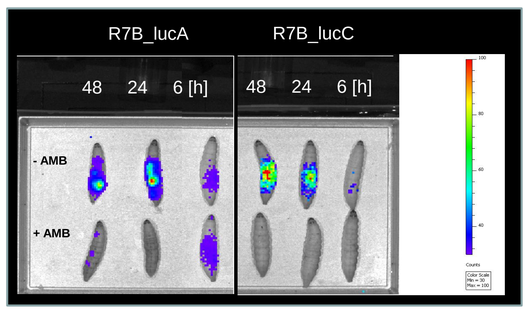
In vivo monitoring of mucormycosis and antifungal efficacy in *Galleria mellonella*



- although light was emitted in efficient amount to be detected in microtiter plate assays, *in vivo* signals were too low to be measured



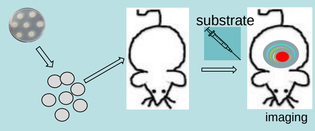
Inoculum: 10^6 spores/larvae
Incubation: 24 - 72 h, 30° C
Luciferin: 60 µL 10 mM Synchem



- codon optimization led to detectable signals in *Galleria mellonella* larvae infected with R7B_lucA and R7B_lucC
- highest signals were obtained in larvae infeccted with R7B_lucC, which harbours Mucor-optimized luciferase under the control of p*his3,* indicating different regulation within the larvae compared to YNB medium
- treatment with 15 mg/kg L-AMB significantly reduced light signals in infected larvae
- bioluminescent reporter strains can be used for testing efficacy of antifungal drugs

Summary and outlook

- successful construction of bioluminescent reporter strains for the first time in the basal fungus *M. circinelloides*
- codon optimization successfully increased light emission & made in vivo imaging of infection possible
- growth, antifungal susceptibility pattern and virulence potential is not altered by the insertion of the luciferase gene under control of any promoter
- generated strains represent a useful tool to study efficacy of (novel) antifungals *in vitro* and *in vivo*, and to monitor disease in a spatial and temporal manner in *Galleria mellonella* & murine models in the future
- use of reporter strains will reduce number of animals in the future
- luciferase will be used for gene expression studies in the future



References: (1) Rodriguez-Frometa et al. Appl Microbiol Biotechnol 2013; (2) Torres-martinez et al. Methods Mol Biol 2012

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