Marshall Plan Scholarship

Final Report

Developing CRISPR/Cas9 in the thermophilic fungus *Thermoascus aurantiacus* for cost-efficient conversion of plant biomass into biofuels and bio-products

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Vienna, 4th of November 2019

Acknowledgements

First of all, I would like to thank the Austrian Marshall Plan Scholarship Foundation which provided me financial support for my stay in the United States of America and I am also thankful for the help I got during the application for the scholarship from Selis Schmidt from the International Office of the University of Natural Resources and Life Sciences Vienna.

Secondly, special thanks go to my principal investigator Steven Singer who gave me the opportunity to work in his research group at the Lawrence Berkeley National Laboratory (LBNL) in California and initiated all organizational steps for my stay abroad. I am grateful that I was part of his research team and that I learned a lot during all the lab meetings.

Besides, I would like to thank my supervisor and mentor Raphael Gabriel for the exceptional experimental guidance through the "*Thermoascus* project" during my 6.5 months stay. He taught me several laboratory techniques, helped me with the experimental design and I highly appreciate his constant support and his patience.

I am also grateful for the help I got from the interns Carlos A. Romero Vazquez and Pallas Chou who both worked with motivation and always kept a cheerful atmosphere in the lab.

I extend my gratitude to every member of the microbial communities group at LBNL and the Joint BioEnergy Institute (JBEI) for the practical help during the work in the laboratory and the social activities after work.

Lastly, I also want to thank Univ.Prof. Dipl.-Ing. Dr.nat.techn. Lukas Mach for his supervision of my Master Thesis and being my referee.

Abstract

Fungal enzymes like cellulases and xylanases can be used to convert non-edible plant biomass to biofuels, bioplastics and various other bio-products. *Thermoascus aurantiacus* is an attractive candidate for that because it is a thermophilic biomass-degrading fungus that produces these enzymes which display higher activity and thermotolerance than currently used enzymes which allow more cost-efficient deconstruction of plant biomass for bio-product generation.

The key for metabolic engineering and unravelling the mechanism of lignocellulose deconstruction is the development of genetic manipulation strategies for *T. aurantiacus*. Since there is a lack of a powerful, versatile genome-editing method the aim of this research work was to develop new genetic tools to alter genes that are involved in cellulases and xylanase production. Therefore, the novel and powerful genome-editing technology CRISPR/Cas9 was established for the first time in *T. aurantiacus* to facilitate genetic modifications. In addition, a classical sexual crossing protocol was developed due to the unique self-fertilizing ability of this fungus.

With these approaches it is possible to gain a fundamental understanding of how enzyme secretion is regulated in this fungus and this knowledge can then be further used to design improved strain and bioprocess engineering strategies for cost-efficient thermostable enzyme production for converting plant biomass into biofuels and bio-products.

In this study, we demonstrated the functionality of the CRISPR/Cas9 system in the filamentous fungus *T. aurantiacus* and the control organism *Aspergillus niger* through *Agrobacterium tumefaciens*-mediated transformation. As a proof of principle, the recyclable marker genes *pyrG* of the *T. aurantiacus* genome and the *albA* gene in *A. niger* were chosen as targets for editing. It was confirmed that the constructed CRISPR/Cas9 vectors generated site-specific mutations in the target *pyrG* as well as in the *albA* gene that were most likely introduced through non homologous end joining events. Additionally, two mutant *T. aurantiacus* strains were sexually crossed, namely a hygromycin-resistant strain with a 5-fluoroorotic acid resistant and uracil auxotrophic strain resulting in a new mutant strain harboring both resistances.

In conclusion, two ways of facilitating genome editing were successfully established for the fungal species *T. aurantiacus* which can be used individually or in combination and have the potential to accelerate strain engineering of the thermophilic fungus to produce industrial enzymes like cellulases and possibly biofuels in the future.

Zusammenfassung

Von Pilzen produzierte Enzyme wie Cellulasen und Xylanasen können verwendet werde um nicht essbare Pflanzenbiomasse zu Biokraftstoffe, Bioplastik und viele andere Bioprodukte umzuwandeln. *Thermoascus aurantiacus* ist ein attraktiver Kandidat dafür, weil es sich dabei um einen thermophilen Pflanzenbiomasse-abbauenden Pilz handelt und die notwendigen Enzyme produziert, welche eine viel höhere Aktivität und Hitzetoleranz aufweisen im Vergleich zu derzeit verwendeten Enzymen. Die Verwendung von diesen *T. aurantiacus*-Enzymen ermöglicht einen kosteneffizienten Abbau von Pflanzenbiomasse zur Herstellung von Bioprodukten.

Der Schlüssel für metabolisches Engineering und um den Mechanismus von Lignocelluloseabbau zu enträtseln, ist die Entwicklung von genetischen Manipulationsstrategien für *T. aurantiacus*. Da es an wirksamen und universell einsetzbaren Methoden um Gene zu verändern fehlt, ist das Ziel dieser Forschungsarbeit die Entwicklung von neuen genetischen Methoden um Gene zu modifizieren welche in der Cellulase- und Xylanaseproduktion involviert sind. Deswegen wurde die neue und wirksame gentechnische Methode CRISPR/Cas9 zum ersten Mal in *T. aurantiacus* etabliert um genetische Veränderungen zu erleichtern. Außerdem wurde ein klassisches sexuelles Kreuzungsprotokoll entwickelt aufgrund der einzigartigen selbstbefruchtenden Fähigkeit des Pilzes.

Mithilfe dieser Methoden kann ein fundamentales Wissen generiert werden, wie Enzymsekretion in diesem Pilz reguliert wird, welches im Weiteren verwendet werden kann um verbesserte Stamm- und Bioprozessengineering Strategien zu designen um kosteneffiziente thermostabile Enzyme zu produzieren welche Pflanzenbiomasse in Biokraftstoffe und andere Bioprodukte umwandeln können.

In dieser Arbeit wurde gezeigt, dass das CRISPR/Cas9 System in dem filamentösen Pilz *T. aurantiacus* und in dem Kontrollorganismus *Aspergillus niger* durch *Agrobacterium tumefaciens*-vermittelte Transformation funktioniert. Als Beweis wurden die beiden rezyklierbaren Markergene *pyrG* im *T. aurantaicus* Genom und *albA* in *A. niger* als Zielsequenzen ausgewählt. Es konnte gezeigt werden, dass durch die konstruierten CRISPR/Cas9 Vektoren spezifische Mutationen in der Zielsequenz *pyrG* als auch in der *albA* Sequenz generiert wurden welche höchstwahrscheinlich durch nichthomologe Endjoining Events erzeugt wurden. Außerdem wurden zwei mutierte *T. aurantiacus* Stämme sexuell gekreuzt, nämlich ein Hygromycin B resistenter Stamm mit einem 5-Fluoroorotische Säure resistenten und Uracil auxotrophen Stamm wodurch ein neuer Stamm resultierte, welcher beide Resistenzen besaß.

Zusammenfassend lässt sich sagen, dass zwei Wege für *T. aurantiacus* etabliert wurden um Genomveränderungen zu erleichtern, welche individuell oder in Kombination eingesetzt werden können und das Potential haben die Erzeugung von neuen gentechnisch veränderten Stämme zu beschleunigen, welche industrielle Enzyme wie Cellulasen und Xylanasen produzieren und möglicherweise auch Biokraftstoffe in der Zukunft.

Abbreviations

°C	Degree Celsius
5-FOA	5-Fluoroorotic acid
A. niger	*Aspergillus niger*
A. tumefaciens	*Agrobacterium tumefaciens*
albA	Polyketide synthase
AS	Acetosyringone
ATMT	Agrobacterium tumefaciens-mediated transformation
CAZymes	Carbohydrate-active enzymes
creA	Carbon catabolite repressor
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
crRNA	CRISPR ribonucleic acid
DNA	Deoxyribonucleic acid
DSB	Double stranded break
dsDNA	Double stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
FAA	Fluoroacetamide
GH	Glycoside hydrolases
gRNA	Guide ribonucleic acid
HDV	Hepatitis delta virus
HH	Hammerhead
hph	Hygromycin-B-phosphotransferase
hyg B	Hygromycin B
IM	Induction medium
LB	Luria-Bertani
M. thermophila	*Myceliophtora thermophila*
NHEJ	Non homologous end joining
OD_{600}	Optical Density 600 nm
P. chrysogenum	*Penicillium chrysogenum*
PAM	Protospacer adjacent motif

PD	Potato Dextrose
PDA	Potato Dextrose Agar
pyrE	Orotate-phosphoribosyltransferase
pyrG	Orotidine-5'-phosphate decarboxylase
rpm	Rounds per minute
S. pyogenes	*Streptococcus pyogenes*
sgRNA	Single guide ribonucleic acid
T. aurantiacus	*Thermoascus aurantiacus*
T. reesei	*Trichoderma reesei*
T-DNA	Transfer DNA
Ti plasmid	Tumour-inducing plasmid
tracrRNA	Trans-activating CRISPR RNA
Vogel's MM	Vogel's minimal medium
WT	Wild-type
YPD	Yeast Extract–Peptone–Dextrose Medium

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1. Introduction

1.1. Scientific background and relevance

Our strong dependence on the usage of fossil fuels leads to major challenges concerning our daily lives. For instance, in terms of transportation, the number of cars and other vehicles are expected to increase within the next years leading to higher petroleum consumption and more emissions of greenhouse gas emissions[1]. To reduce further impacts of global warming, there is an urgent need for environmentally friendly alternatives like solar, wind or geothermal energy amongst others[2]. Another approach to substitute fossil energy is the use of non-edible lignocellulosic biomass which is the most abundant organic polymer on our earth. It is renewable and does not release carbon dioxide into our atmosphere[1,3]. Since non-edible cellulosic plant biomass residues are also a cheap resource available in large amounts from agriculture and forestry, they can be used to be converted into virtually any product from biofuels to bioplastics, textiles, medication and other products needed in our daily lives, with the potential of replacing petroleum[4,5]. Besides, the conversion of waste products to clean energy is a promising way to address the energy crisis we are currently facing, thus the biofuel research is a crucial step towards this energy change[1].

Organic plant biomass consists mainly of lignocellulose which consists of the polymers cellulose, hemicellulose and lignin. Cellulose makes up to 60 % of the plant and is a long-chained sugar consisting of β-1,4-linked D-glucose molecules and gives structure and mechanical strength to plant cell walls[2]. The conversion of plant biomass can be performed by microorganisms which produce certain enzymes, namely cellulases and xylanases, which have the natural ability to break down plant biomass into its chemical building blocks which are mainly simple sugars like glucose. These sugar molecules can then be converted to basically any product through metabolically engineered microbes like yeasts and bacteria[6]. This makes plant-based biomass more versatile in comparison to conventional renewable energy sources but it is still an expensive approach and the production costs of the cellulases and xylanases required for the process is one of the limiting factors[4,7].

Filamentous fungi naturally produce large amounts of enzymes that break down plant biomass but the currently produced enzymes are not very thermostable[8]. Plant biomass deconstruction at higher temperatures is not only less prone to contamination but also enables higher enzymatic reaction rates, better flow characteristics of biomass slurry and reduces necessary cooling of the emerging process heat, which reduces the overall process costs in the end[9]. Hence, there is a high demand for the generation of novel fungal strains with advanced properties that can successfully degrade plant biomass[10]. The thermophilic ascomycete fungus *Thermoascus aurantiacus* is an excellent producer of cellulase and xylanases that are more heat-stable and active compared to enzymes from currently used industrial fungi and is therefore a promising candidate for establishing and improving the potential of this fungus as a thermostable cellulase production platform[11,12].

1.2. The ascomycete fungus *Thermoascus aurantiacus*

Ascomycete fungi possess the ability to produce lignocellulosic enzymes that are able to deconstruct plant biomass to use it for biofuels and other bio-product production and are therefore predominantly used for the production of commercial enzymes[13,14]. They release high amounts of carbohydrate-active enzymes (CAZymes), which degrade polysaccharides and the converted simple sugars provide them energy. The secreted enzymes not only depend on the available carbohydrates in the environment but also on the fungal species and the secretion is controlled by transcription regulators. Currently, ascomycete fungi like *Trichoderma reesei* produce most cellulases that are commercially available. Further knowledge about the regulations of fungal CAZymes in combination with biotechnological methods is needed to generate new hypersecretory fungal strains that reduce the costs for breaking down cellulose[15].

A promising approach is to use thermophilic fungi as producers to develop platforms for cellulose production. *T. aurantiacus* is an interesting fungal species that secretes thermostable enzymes for plant biomass deconstruction[9]. It is a thermophilic ascomycete fungus within the order of the *Eurotiales* and was isolated for the first time by Hugo Miehe in 1907 from self-heating hay, but later also from terrestrial habitats like compost, soil or wood chips[14,16]. The new genus *Thermoascus* (thermos [greek] = hot, askos [greek] = tube) was generated and due to the orange coloured fruiting bodies of the fungus it was assigned to the species *aurantiacus* (lat., orange). The fruiting bodies consist of cleistothecia which contain asci with eight ascospores (Figure 1). The sexual ascospores are produced in high amounts by *T. aurantiacus* and are believed to be the dominant form of propagation whereas asexual conidia are present in most ascomycete fungi but are not present in *T. aurantiacus*. Although asexual chlamydospores were found in *T. aurantiacus* no germination has ever been discovered[14]. Important to mention is that *T. aurantiacus* is a homothallic fungal species, meaning that it is able to mate with itself and this feature can be exploited through the establishment of sexual crossing strategies which is only possible in few fungi since most ascomycete fungi reproduce asexually. Sexual crossing procedures can be used as alternative to classical mutagenesis approaches to develop quick methods for improved strain engineering[17]. The optimal growth temperature of *T. aurantiacus* was defined to be at 50 °C whereas no growth is possible below 30 °C. The growth speed and the sexual reproduction cycle is remarkably fast compared to other thermophilic fungi[14].

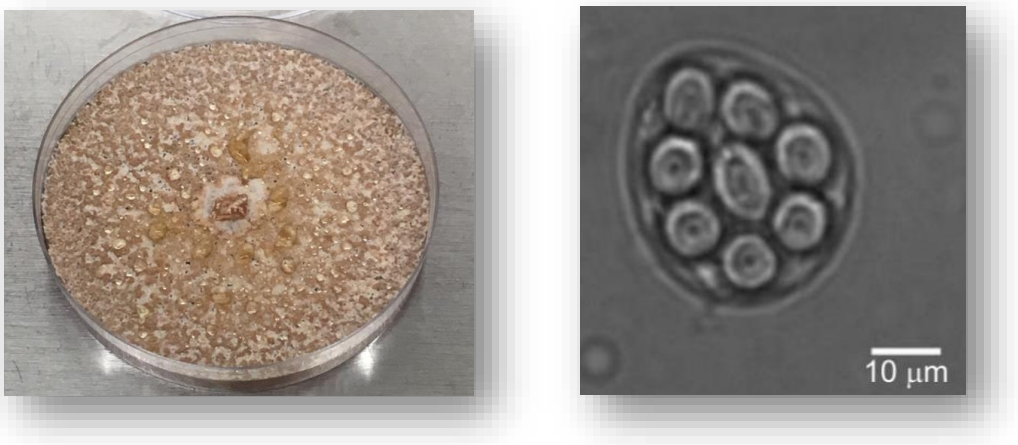

Figure 1: Left: T. aurantiacus ATCC 26904 wild-type strain on TEKNOVA Potato Dextrose Agar (PDA) plates incubated for 2 days at 50 °C and 4 days at 45 °C. Right: T. aurantiacus ascus with eight ascospores in it (Schuerg et al[14]).

It was observed that *T. aurantiacus* WT strain ATCC 26904 produces high levels of cellulases, more than 2 g/L including the following four glycoside hydrolases: beta-glucosidase (GH3 / LPMO), cellobiohydrolase (GH7), endoglucanase (GH5) and lytic polysaccharide monooxygenase (GH61) which remarkably still show activity at high temperatures above 50 °C compared to the commonly used commercial enzyme mix CTec2 by Novozymes[18]. The former enzymes were shown to be ideal for efficient breakdown of plant biomass to the biofuel butanol[11].

All these features make the fungus *T. aurantiacus* a promising candidate for studying sources of enzymes to improve hydrolysis of lignocellulosic biomass. That is why it is necessary to work amongst others on genetic tools development and optimization strategies in order to generate improved *T. aurantiacus* strains through modification of genes responsible for cellulase production to develop an industrial thermophilic cellulase producer[14,18].

1.3. Genetic tools development for use in filamentous fungi

The focus on performing genomic research work on filamentous fungal species is of importance in order to gain knowledge about the genetic regulations and functions of genes in these fungi as well as to perform genetic studies. The establishment of new transformation and gene manipulation strategies is of high interest to generate improved strains for diverse biotechnological applications[19]. Although genetic engineering methods have already been developed for filamentous fungi like *T. reesei*, *Aspergillus niger* or *Penicillium chrysogenum*, the deletion of specific genes is still very tedious and time consuming. However, being able to perform efficient gene deletions is the key to further improve the potential of filamentous fungi like *T. aurantiacus* for increased enzyme production. The development of a gene-editing system as well as a sexual crossing protocol can be used to facilitate the work with *T. aurantiacus* and establish this fungus as a potent cell factory for lignocellulolytic enzyme preparations and also to uncover the regulatory mechanisms for induction and secretion of proteins in *T. aurantiacus*[20].

1.3.1. Selectable marker genes

In order to develop genetic tools and facilitate the screening process of positive transformants the development and use of different selection markers, like antibiotic resistance genes, is of high importance[21]. For instance, the introduction of a *hph* gene cassette into a eukaryotic cell is often performed because this gene encodes a hygromycin-B-phosphotransferase (*hph*) and its expression leads to hygromycin B (hyg B) resistance through inactivation of the antibiotic by phosphorylation[22].

Other genes that are widely used as selectable and also counter-selectable marker in model fungal species are the *pyrG and pyrE* genes[21].The *pyrG* gene encodes orotidine-5'-phosphate decarboxylase whereas *pyrE* encodes orotate-phosphoribosyltransferase, both genes are involved in the pyrimidine biosynthetic pathway with is vital for uridine synthesis. Deletion of either of the two genes results in mutant strains that show resistance to the toxic compound 5-fluoroorotic acid (5-FOA) and are uracil auxotrophic which can be selected on cultivation media supplemented with uracil and 5-FOA[23].

Deletion of the *albA* gene in *A. niger* can be exploited as a phenotypic marker since *albA* is responsible for the black spore pigmentation of this fungus. Inactivating this gene results in pale conidia. Thus, successful transformants can easily be screened for due to the resulting pale conidia of an inactive *albA* allele instead of the characteristic black coloured conidia spores of the *A. niger* WT strain[24].

1.3.2. *Agrobacterium tumefaciens*-mediated transformation

The natural ability of the gram negative soil bacterium *A. tumefaciens* to transfer deoxyribonucleic acid (DNA) segments into plants and also fungi can be exploited to generate desired knockout strains of filamentous fungi through *Agrobacterium tumefaciens*-mediated transformation (ATMT)[25,26]. *A. tumefaciens* is a plant pathogen that infects wound sites of infected plants and causes tumour growth. It has in addition to its bacterial chromosome a 200 bp tumor-inducer plasmid (Ti plasmid) with a replaceable transfer DNA (T-DNA) that can be randomly inserted into the plant chromosomes via gene transfer as depicted in Figure 3. This T-DNA can be easily genetically modified with the gene of interest. The transfer is initiated by expressed virulence genes on the Ti plasmid which are activated by the chemical compound acetosyringone (AS) released by the wounded plants[27]. Since this insertion of foreign genes mediated by the natural genetic engineer *A. tumefaciens* also works in fungi, a successful ATMT protocol was recently established by JBEI researchers and optimized for use for *T. aurantiacus* (unpublished data).

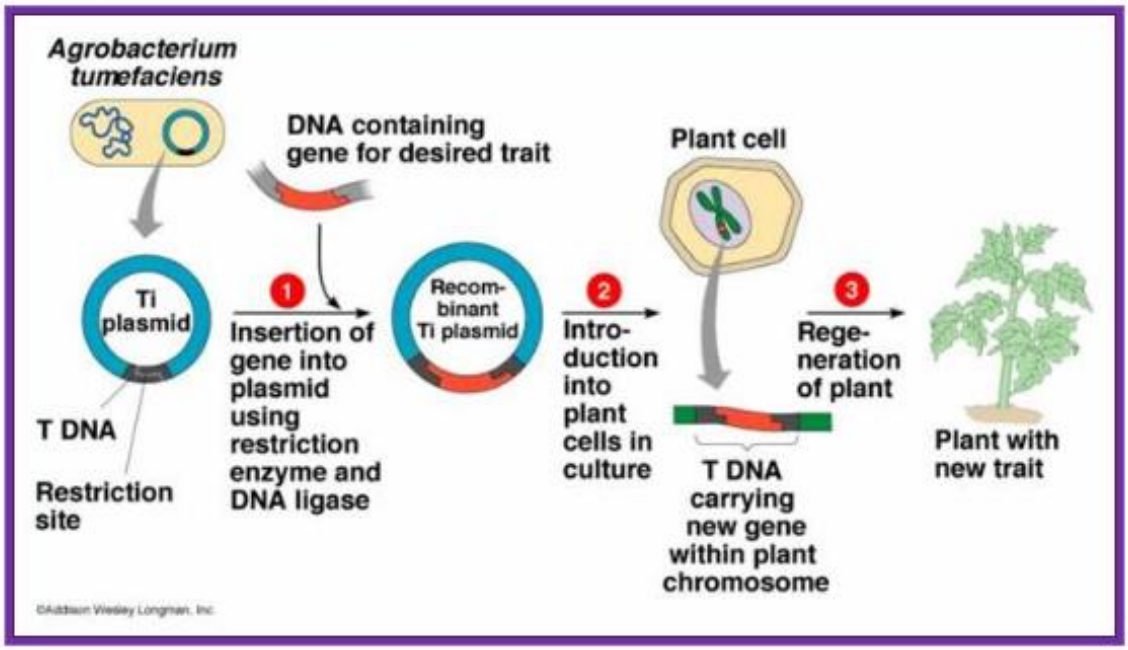

Figure 2: Overview of natural genetic engineering capability of A. tumefaciens for use for ATMT in filamentous fungi. (Source: http://plantcellbiology.masters.grkraj.org/html/Genetic_Engineering4D-Transformation-Plant_Cells.htm)

1.3.3. CRISPR/Cas9 gene editing system

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9) system, short CRISPR/Cas9, is a novel genetic engineering tool that can facilitate the site-specific deletion and modification of genes in many different organisms and this technology can be used for fungi in order can be used to create new fungal strains and get insight into the genetic regulation of those genes[20]. CRISPR/Cas9 is a promising type II gene modification technology and part of a bacterial immune system where two components work together to destroy virus DNA in the cell, namely the DNA cleaving endonuclease Cas9 with a single guide ribonucleic acid (sgRNA) as depicted in Figure 2. The Cas9 endonuclease introduces a double stranded break (DSB) in the target DNA sequence which is complementary to the 20 bp protospacer sequence of the sgRNA[28]. The full length guide RNA (gRNA), which is a fusion of CRISPR ribonucleic acid (crRNA) and Trans-activating CRISPR RNA (tracrRNA), is necessary for CRISPR/Cas9-mediated genome editing because it directs the Cas9 endonuclease to the target sequence which it can specifically bind to and cleave by creating a DSB in the DNA. The repair of the introduced DSB is most likely performed through non homologous end joining (NHEJ) which leads to mutations and therefore the knockout of the target gene. One restriction of the system is the necessity of the protospacer adjacent motif (PAM) which has to be present adjacent to the binding site of the protospacer sequence at the target sequence. The PAM depends on the used Cas9 enzyme. *Streptococcus pyogenes* Cas9 is mostly used for CRISPR/Cas9 genome editing, thus a NGG sequence is accepted as PAM[21].

This CRISPR/Cas9 technology provides great potential as a tool for genetic engineering in filamentous fungi due to the site specificity of the Cas9 endonuclease activity and the simplicity of gRNA construction[20]. By changing the sequence of the gRNA, Cas9 can be directed to novel

targets to delete any genes of interest. Both, the Cas9 gene and the gRNA gene can be randomly integrated with ATMT into fungal genomes, which then leads to high-efficiency deletion of target genes upon expression of Cas9 and the gRNA. A variant of the CRISPR/Cas9 technology is the so-called ribozyme-based release strategy which was designed for gene editing reasons of filamentous fungi[29]. In that case, the sgRNA is in the middle of a large transcript which is synthesized by RNA-polymerase II and liberated through the self-splicing ability of two ribozyme sequences flanking it, namely the 5'end hammerhead (HH) and 3'end hepatitis delta virus (HDV). Using this technology basically any promoter can be used for expression and there is no need for messenger RNA polymerase III promoter which are ill-defined in filamentous fungi[21].

The CRISPR/Cas9 technology is the most popular genetic engineering system so far and it even possesses multiplexing capability, meaning that more than one gene can be deleted simultaneously which makes genome editing less time- and labour-consuming. This is a major benefit over previously developed strategies like zinc-finger nucleases since they require certain enzymes and the process takes very long[20]. The CRISPR/Cas9 system has already been used in *Myceliophtora thermophila* to delete multiple genes repressing cellulase secretion which increased the cellulase activity 7-fold and xylanase activity 12-fold[28].

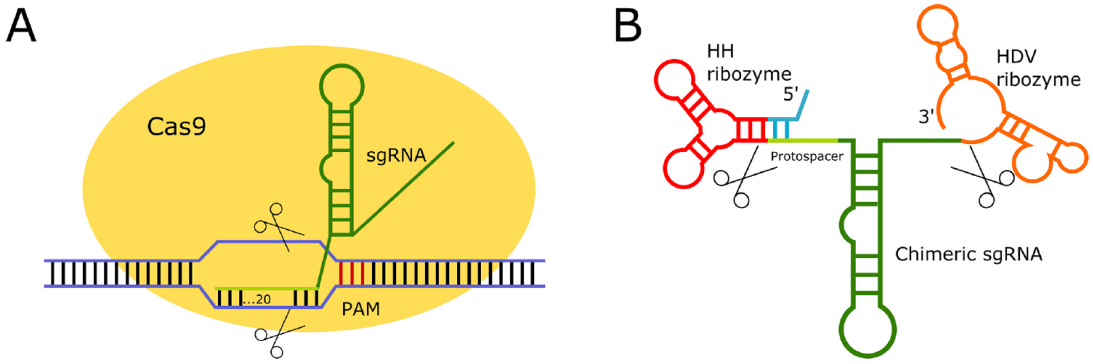

Figure 3: CRISPR/Cas9 gene-editing system from Nodvig et al[21]. Components of the CRISPR/Cas9 system are depicted in A. Cas9 endonuclease cleaves the target sequence through sgRNA guidance (depicted in green). The protospacer sequence of the sgRNA is shown in light green and base pairs with the target sequence that is downstream of the PAM sequence. Double stranded cleavage happens 3–5 bp upstream of the PAM which is marked with scissors. Ribozyme based release strategy depicted in B. HH ribozyme and HDV ribozyme liberate the sgRNA through self-cleavage marked with scissors. This figure was created by Nodvig et al[21].

1.3.4. Sexual crossing tools

Although sexual reproduction is the dominant form of breeding in eukaryotes, many industrially used fungi do not reproduce sexually, hence classical mutagenesis approaches are used for industrial strain improvement while sexual strain crossing methods are not available. However, sexual crossing can be advantageous because crosses of fungal isolates with different resistance markers against antibiotics enables the generation of new strains with new characteristics for simple screening purposes that facilitate the work with those fungal species[17]. Compared to

other methods like random mutagenesis, the sexual crossing procedure can be faster and cheaper for multiple gene modifications and this method does not lead to genetic instabilities of the crossed strains[30]. So far, sexual crossing tools are hardly available for filamentous fungi and we hypothesized that sexual reproduction can be used in *T. reesei* opening new ways of strain engineering for industrial applications[17]. A remarkable feature of *T. aurantiacus* is the self-fertilizing ability which can be exploited through the development of a sexual crossing protocol by crossing two mutant strains resulting in a new strain producing genetically identical spores. Besides, the reproduction cycle is completed within six days which is quite fast in comparison to other fungal species. Thus, this approach seemed to be promising for designing advanced strain engineering strategies for improved plant biomass conversion to biofuels and other bio-products[14].

1.4. Aim of the thesis

The overall goal is the development of the CRISPR/Cas9 genome editing system for the first time in the thermophilic fungus *T. aurantiacus* in order to expand the knowledge of the cellulase regulation in this fungus and to design improved strain engineering strategies for cost-efficient cellulase production to help generate biofuels and other bioproducts from plant biomass. With the help of the previous establishment of the ATMT protocol of *T. aurantiacus* it is currently possible to randomly integrate genes of interest into the fungal genome while the deletion of specific genes is still very tedious. Being able to perform efficient gene deletions is the key for further improving the potential of this fungus for enzyme production. With the CRISPR/Cas9 technology, gene modifications can be rapidly introduced simply by changing the sequence of the sgRNA to target and delete any gene of interest upon correct integration and expression of Cas9 and the gRNA in the fungal genome. Besides, a classical sexual crossing protocol will be established due of the unique self-fertilizing ability of *T. aurantiacus* in order to develop more tools for better strain engineering and generate a fundamental understanding of how *T. aurantiacus* regulates enzyme production and uncover synergies between modified genes for improving cellulase secretion for biofuel production from lignocellulose.

This strategy comprised the following two phases (Figure 4). The first phase deals with the construction of new ATMT CRISPR/Cas9 vectors containing gRNAs to target the *pyrG* gene and the *amdS* gene in *T. aurantiacus* and verify that the DNA sequences contain no errors. After that, these vectors will be used in Phase II to integrate the Cas9 and the gRNA genes into the fungal genome and select for transformants with a deleted *pyrG* and *amdS* gene first on hyg B plates and further on 5-FOA and fluoroacetamide (FAA) plates respectively. Verification of insertion of the integrated DNA as well as the deletion of the target genes will be performed with PCR.

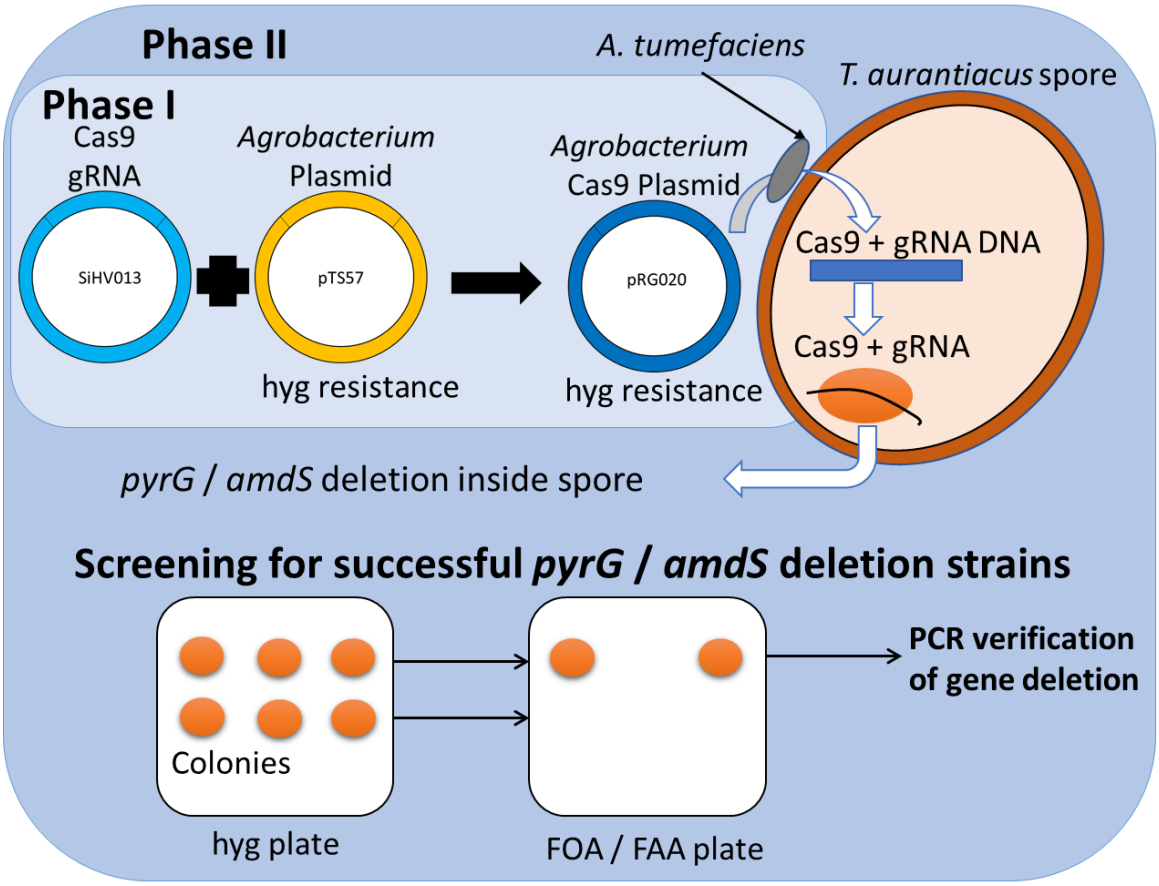

Figure 4: Overview of the proposed gene editing work flow via CRISPR/Cas9. In Phase I, plasmid containing the Cas9 gene and a cloning site for the gRNA will be generated from the precursor plasmid pTS57 which will be used in Phase II to knock out the native pyrG and amdS gene of T. aurantiacus. All transformants that grow on hyg B plates will be plated again on 5-FOA/ FAA plates that select for strains with successful pyrG or amdS deletion respectively, which will be verified with PCR. (Raphael Gabriel)

2. Materials and methods

2.1. Fungal strains and culture conditions

T. aurantiacus WT strain ATCC 26904 was obtained from the American Type Culture Collection and grown on solid TEKNOVA Potato dextrose agar (PDA) plates for transformation purposes. The PDA plates were inoculated with three- to six-day-old ascospores and incubated for two days at 50 °C before they were transferred to 45 °C for another four days. This shift was performed due to harsh evaporation of PDA plates at 50 °C. The plates were covered with a glass beaker to reduce drying and plastic containers filled with distilled H_2O at the bottom of the incubators provided a moist atmosphere.

The four *T. aurantiacus* strains 45-1, 45-2, 45-3 and 45-4 were previously isolated after random UV mutagenesis for 45 seconds in order to obtain strains with 5-FOA resistance and uracil auxotrophy. Cultivation of these strains was usually performed on solid Vogel's minimal medium (Vogel's MM) with 2 % sucrose and 1.5 % bacto agar supplemented with 1 g/L uracil and 1 g/L 5-FOA. For Vogel's salts solution preparation see appendix.

Mutant *T. aurantiacus* strain taRG008 was previously generated through ectopic integration of a *hph* gene cassette via the ATMT procedure as later described in section 2.5. and is therefore

resistant to the antibiotic hyg B. The strain was usually grown on PDA medium supplemented with 50 µg/mL hyg B.

A. niger WT strain ATCC 1015 served as control organism in this study and was streaked out on slanted PDA medium. The incubation conditions were 30 °C for six days to ensure the same incubation time as for *T. aurantiacus*.

Fungal mycelia for subsequent genomic DNA extraction was grown in liquid potato dextrose (PD) broth supplemented with 1 g/L uracil when required or in Vogel's MM with 2 % sucrose, 0.8 % soy peptone and uracil (1 g/L) when required through inoculation with six-day-old *T. aurantiacus* ascospores. The cultivation was performed in a 1 mL volume at 45 °C for three days in a 24-well plate and 180 rpm for the first transformation round and then the culture conditions were changed to a 3 mL volume in sterile glass vials at 37 °C for three days on a shaker since the latter approach is less prone to cross contamination.

A. niger conidia were inoculated in 3 mL liquid PD broth in glass vials and cultivated at 30 °C for three days at 200 rpm.

All strains used for sexual crossing and ATMT experiments are listed in Table 1 and Table 2.

Table 1: T. aurantiacus strains used in this study for sexual crossing experiments

Strain name	***T. aurantiacus***	**Resistance**
ATTC 26904	WT	No resistance
45-1	Mutant strain	5-FOA
45-2	Mutant strain	5-FOA
45-3	Mutant strain	5-FOA
45-4	Mutant strain	5-FOA
taRG008	Mutant strain	Hyg B

Table 2: Fungal strains used in this study for CRISPR/Cas9 ATMT experiments

Fungal species	**Strain name**	**Target gene**	**Introduced plasmid via ATMT**	**Bacterial resistance**	**Fungal resistance**
T. aurantiacus	ATTC 26904	*pyrG*	pJP1 pJP3	kanamycin	Hyg B 5-FOA
A. niger	ATCC 1015	*albA*	pJP4	kanamycin	Hyg B

2.2. Spore isolation and counting

Ascospores of *T. aurantiacus* were harvested twice in 5 mL 0.8 % Tween80 solution by scraping them off with a disposable cell spreader and filtering them through a P1000 tip with Miracloth for subsequent transformation purposes. Conidia from *A. niger* were harvested only once in 3 mL 0.8 % Tween80 solution. For the determination of the number of spores 10 µL of

the undiluted or 1:100 in distilled H_2O diluted spore suspension was loaded on both plateaus of the Bright-Line hemocytometer below the cover glass. The spores were counted on both sides within the counting grid which consists of 25 groups of 16 small squares each, where each group is separated by triple lines (see Figure 5) using a light microscope. Then the mean was calculated from both spore counts and the following equation was used to calculate the number of spores per milliliter:

$$\text{number of spores/mL} = \text{mean of nr. of counted cells} \times \text{dilution factor} \times 10000$$

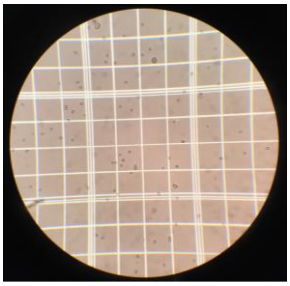

Figure 5: Counting grid of one group consisting of 16 small squares, each group separated by triple lines visualized with Vista Vision microscope using a 40X objective.

For transformation purposes a total of $1*10^8$ *T. aurantiacus* ascospores were used. For *A. niger* a total of $2*10^6$ spores were used for ATMT.

2.3. Cloning hosts for plasmid propagation

Escherichia coli strain DH5α (NEB, Ipswich, MA, United States) was used for vector propagation and was cultivated at 37 °C in liquid or solid Luria-Bertani (LB) medium supplemented with 100 µg/mL kanamycin or carbenicillin, which was substituted for ampicillin, for selection reasons.

Newly constructed plasmids were transformed into chemically competent 5-alpha Competent *E. coli* cells (NEB, Ipswich, MA, United States) via heat shock following the Gibson Assembly Chemical Transformation Protocol (NEB, Ipswich, MA, United States). Plasmid DNA was obtained through purification of 8 mL of an overnight culture using QIAprep Spin Miniprep Kit 250 (Qiagen, Hilden, Germany). Elution was performed with nuclease free water. The DNA concentration and the purity coefficient were measured with NanoDrop2000.

For transformation experiments mediated by *A. tumefaciens* the previously constructed *E. coli* vectors were transformed into electrocompetent *A. tumefaciens* EHA105 cells via electroporation. Electrocompetent *A. tumefaciens* stocks were obtained by cultivating 1 mL of an overnight culture (at 30 °C and 200 rpm) in fresh 100 mL LB medium until an Optical Density (OD_{600}) of 1 was reached at a wavelength of 600 nm. The culture was then transferred into a 50 mL Falcon tube and the cells were pelleted by centrifugation at 4000 rpm for 15 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 25 mL ice cold 1 mM HEPES buffer (pH = 7). Centrifugation was performed as described above. The supernatant was discarded and the pellet was resuspended in 2 mL ice cold 1 mM HEPES buffer with 10 % glycerol (pH = 7) followed by centrifugation. After discarding the supernatant the pellet was resuspended in 0.5 mL ice cold 1mM HEPES buffer with 10 % glycerol (pH = 7).

Aliquots of 50 µL were pipetted into sterile 1.5 mL Eppendorf tubes and the electrocompetent *A. tumefaciens* aliquots were stored at -80 °C.

For electroporation, electrocompetent EHA105 *A. tumefaciens* cells were thawed on ice before 50 µL were combined with 1 µL of 10 ng/µL diluted plasmid DNA and incubated for about 2 minutes on ice. Then the cell-DNA mixture was transferred into a chilled sterile 2 mm VWR electroporation cuvette and the mixture was tapped to the bottom of the cuvette. The Gene Pulser Xcell™ Electroporation System (BioRad, Hercules, CA, United States) was set to 25 µF capacitor, the pulse controller unit was set to 400 ohms and a single 2.5 kV electrical pulse was applied with a field strength of 12.5 kV/cm. After the pulse 1 mL of LB broth was immediately added to the cuvette in which the cells were gently resuspended. The cell suspension was transferred to a 15 mL tube and incubated at 30 °C and 200 rpm for about 2 hours.
After that, 300 µL of each plasmid were plated on LB agar plates containing 50 µg/mL of the antibiotic kanamycin and incubated at 30 °C. Transformed colonies were expected after 2 - 3 days.
All ATMT experiments were performed with *A. tumefaciens* strains carrying the correct plasmid which were streaked out onto LB agar plates containing the appropriate antibiotic. After incubation for two to three days at 30 °C, a single *A. tumefaciens* colony was randomly picked and used for inoculation of 10 mL liquid LB-kanamycin medium in order to select against the loss of the plasmid at 30 °C and 200 rpm overnight.

All used host strains and plasmids used in this study are listed in Table 3.

Table 3: Host strains and plasmids used in this study

Host organism	**Plasmid name**	**Bacterial resistance**	**Use**	**Fungal resistance**	**Target gene**
DH5α *E. coli*	Pllk003 (pFC332)	ampicillin	USER Cloning	/	/
DH5α *E. coli*	Pllk006 (pFC336)	ampicillin	USER Cloning/ Gibson Assembly	/	/
DH5α *E. coli*	JP36_1	ampicillin	Gibson Assembly for pJP1	/	/
DH5α *E. coli*	JP36_3	ampicillin	Gibson Assembly for pJP3	/	/
DH5α *E. coli*	pTS57	kanamycin	Gibson Assembly for pJP1/pJP3	/	/
DH5α *E. coli*	pJP1	kanamycin	Electroporation into *A. tumefaciens*	/	/

Host organism	Plasmid name	Bacterial resistance	Use	Fungal resistance	Target gene
DH5α *E. coli*	pJP3	kanamycin	Electroporation into *A. tumefaciens*	/	/
DH5α *E. coli*	pJP4	kanamycin	Electroporation into *A. tumefaciens*	/	/
EHA 105 A. tumefaciens	pJP1	kanamycin	ATMT into *T. aurantiacus*	Hyg B 5-FOA	*pyrG*
EHA 105 A. tumefaciens	pJP3	kanamycin	ATMT into *T. aurantiacus*	Hyg B 5-FOA	*pyrG*
EHA 105 A. tumefaciens	pJP4	kanamycin	ATMT into *A. niger*	Hyg B 5-FOA	*albA*

2.4. Plasmid design and cloning strategy

New CRISPR/Cas9 plasmids carrying the two necessary components for the CRISPR/Cas9 system, namely the Cas9 nuclease as well as the sgRNA, were designed to target the *pyrG* gene in the target host *T. aurantiacus* and the phenotypic marker gene *albA* in *A. niger* in order to introduce DSBs in the DNA of the target sequence. The target sequences of *T. aurantiacus* and *A. niger* were obtained from JGI (https://mycocosm.jgi.doe.gov/mycocosm/home).

All plasmid maps were designed using the software Geneious 11.1.2 (https://www.geneious.com). All primers were designed using the online tool NEBuilder (https://nebuilder.neb.com). All primer sequences used in this study were ordered from IDT and are listed in the appendix table.

2.4.1. Protospacer design

The gRNA contains a 20 bp long variable sequence which can be changed to target any DNA sequence that is upstream of the NGG PAM in the target sequence. All sgRNAs used in this study were designed using the CRISPOR algorithm (http://crispor.tefor.net) to obtain predicted guide sequences for PAMs in the target gene. For the sgRNA design targeting *pyrG* in *T. aurantiacus* (jgiTheau1), three different gRNA sequences (protospacers) with zero off-target were chosen and tested *in vitro* before performing *in vivo* transformation experiments.
A sgRNA targeting *albA* in *A. niger* was not newly designed because the obtained vector pllk006 (originally pFC336 from Addgene) already contained a guide RNA targeting the gene of interest. Table 4 presents all protospacer sequences with their corresponding PAM sequence and target locus used in this study.

Table 4: List of protospacer and PAM sequences of each target locus used in this study

Target locus	sgRNA name	Protospacer sequence	PAM
pyrG	sgRNA1	CTTTTGCGCGCGAGCGCCGT	AGG
pyrG	sgRNA2	TCGGCGCCCGACTTCCCCTA	CGG
pyrG	sgRNA3	TCGGCGCCCGACTTCCCCTA	CGG
albA	sgRNA4	AGTGGGATCTCAAGAACTAC	TGG

2.4.2. gRNA synthesis and in vitro Cas9 cleavage assay:

Three sgRNAs targeting *pyrG* in *T. aurantiacus* were tested for correct cleavage of the target sequence by Cas9 endonuclease *in vitro*. All steps for the gRNA generation, which consists of the assembly of the gRNA DNA template, the *in vitro* transcription of the DNA templates into RNA and the purification of the transcribed RNA, were performed according to the GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States).

Then the *in vitro* Cas9 cleavage was performed using previously amplified target *pyrG* amplicon. The steps were performed following the Guide-it™ sgRNA In Vitro Transcription and Screening Systems User Manual (Takara Bio USA, Inc., Mountain View, CA, United States). A 2 % Agarose Gel electrophoresis was performed to reveal Cas9 cleavage in the target *pyrG* gene sequence.

2.4.3. Cas9 plasmid construction via USER cloning

For the CRISPR/Cas9 vector construction the established CRISPR/Cas9 mediated genetic engineering system designed for use in filamentous fungi described by Nodvig et al[21] was followed. The aim was to generate plasmids carrying a specific Cas9 gene and a sgRNA targeting the gene of interest through USER Cloning mediated by *E.coli*.

The two *A. niger* vector backbones intended to be combined via USER Cloning were obtained from Addgene (http://www.addgene.org). Vector pllk003 (originally pFC332) carries a Cas9 gene from *S. pyogenes* which was codon optimized for expression in *A. niger* and is followed by a sequence that encodes a SV40 nuclear localization signal, both embedded between the strong constitutive tef1 promoter and tef1 terminator. Besides it carries an *hph* gene cassette for selection as well as an AMA1 sequence responsible for replication in different fungi. Sticky ends necessary for USER cloning were obtained through restriction enzyme digestion of PacI and Nt.BbvCI.

The second vector, pllk006 (originally pFC336) used for USER Cloning contains a sgRNA gene with a protospacer sequence that targets the *albA* gene in *A. niger* under the control of the strong constitutive *Aspergillus nidulans gpdA* promoter and *trpC* terminator. The sgRNA is expressed by two self-splicing ribozymes that flank it, namely the 5'end HH and 3'end HDV. Due to this release technology mediated by the two ribozymes there are two variable regions in the sgRNA gene which both had to be amplified via PCR and were then fused and inserted into the USER cassette of the pllk003 plasmid. The first PCR fragment contains the protospacer sequence with 20 bp, the other one contains a 6 bp sequence that is the reverse complement of

the protospacer which is necessary in the HH sequence for the HH cleavage. Primers had to contain a tail with an uracil base for USER Cloning which were designed according to the paper from Nodvig et al[21] and are listed in the appendix primer table. Phusion U Hot Start DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, United States) was used for amplification of the fragments. Elimination of the uracil bases in the PCR fragments was achieved through USER Enzymes (NEB, Ipswich, MA, United States) which resulted in complementary overhangs at the end of all fragments. After amplification, the two PCR fragments were fused in a directional manner and inserted into vector pllk003 by USER Cloning in a single step following the NEB USER Enzyme protocol.

With this method two plasmids were constructed with a Cas9 gene cassette and a gRNA gene targeting *pyrG* in *T. aurantiacus*, the protospacer and the reverse complement sequence was replaced with the *in vitro* tested sgRNA (sgRNA1 and sgRNA3) sequences. The resulting plasmids were named JP36_1 and JP36_3 respectively. The constructed plasmids were then transformed into NEB 10-beta competent *E. coli* cells, positive transformants were selected for ampicillin resistance on LB-carbenicillin plates (100 µg/ mL carbenicillin). All steps were performed according to the High Efficiency Transformation Protocol using NEB 10-beta Competent E. coli (High Efficiency) (C3019).

Correct plasmid assembly was verified via colony PCR using NEB Phusion High-Fidelity DNA Polymerase. Therefore, ten single colonies were randomly picked and used as DNA for amplification of the *hph* gene from the original pllk003 plasmid as well as the sgRNA gene (sgRNA1 and sgRNA3 respectively) from the original pllk006 plasmid. After obtaining bands of the expected length on a 1 % Agarose Gel the purified PCR products were sequenced by sending the samples to the external company Genewiz for Sanger sequencing analysis. Additionally, the in-house DIVA DNA sequencing service was used to sequence the whole plasmids JP36_1 and JP36_3.

2.4.4. Gibson assembly

In order to construct the new CRISPR-Cas9 vector that can be introduced into fungal cells via the ATMT procedure described in chapter 2.5., the sgRNA-Cas9-gene-cassette from the previously assembled JP36_1/ JP36_3 plasmids had to be inserted into an *Agrobacterium* vector backbone. Therefore, the molecular cloning method Gibson Assembly was used to join two DNA fragments with overlaps under isothermal conditions according to NEB Gibson Assembly Cloning Kit. Plasmid pllk006 harboring a sgRNA targeting the *albA* gene in *A. niger* was also used for Gibson Assembly to construct the control plasmid for ATMT into *A. niger*. Vector pTS57 was used which was especially designed for ATMT into *T. aurantiacus* and harbors a *hph* gene under the control of the Ptef1 promoter and *TtrpC* terminator for selection on hyg B plates as well as the necessary elements for *Agrobacterium*-mediated transformation, namely the transfer DNA between the left and right border, which is replaceable.

Specific primers were designed and used with NEB Q5 Hot Start High-Fidelity DNA Polymerase to amplify the gene cassettes of JP36_1, JP36_3 and pllk006 reaching from the gpdA Promoter to the Tef1 terminator which includes the Cas9 and gRNA genes, as well as the fragment of the vector pTS57 reaching from the left border to the Ttrpc terminator. A restriction

enzyme digest with DpnI was performed to destroy the plasmid template before setting up the assembly reaction. DpnI cleaves only *E. coli* Dam methylase-methylated plasmid DNA but does not cleave the PCR product since it is not methylated.
A 1 % Agarose Gel electrophoresis was performed to prove correct amplification of the fragments. The annealing temperature was chosen according to the NEB Tm Calculator (https://tmcalculator.neb.com). The correct bands were then cut out from the gel under UV light, the fragment DNA was extracted from using QIAEX II Gel Extraction Kit and the DNA concentrations were measured with NanoDrop2000 before the fragments were joined via Gibson assembly using NEB Gibson Assembly Cloning Kit 5510S. The steps were performed according to the NEB Gibson Assembly® Protocol. The necessary pmol amounts of the fragments for Gibson assembly were calculated with the following formula:

$$\text{pmol} = \frac{\text{weight in ng} \times 1000}{\text{base pairs} \times 650 \text{ daltons}}$$

For plasmid construction of pJP1, pJP3 and pJP4 a 1:1 ratio was chosen since the product of both fragments were approximately of the same length. A positive control which was ampicillin resistant was performed, too. The incubation time at 50 °C was extended to 1 hour.
The assembled plasmids were then transformed into chemically competent *E. coli* cells following SIG10 5-alpha Chemically Competent Cells the protocol and selected on LB Agar plates supplemented with 50 µg/mL kanamycin by incubation at 37 °C overnight. The next day a mini prep was performed using QIAprep Spin Miniprep Kit in order to use the DNA for PCR verification of the newly assembled plasmid named pJP1 and pJP3 harboring sgRNA1 and sgRNA3 targeting *pyrG* respectively as well as pJP4 harboring a sgRNA targeting the *albA*. The *hph* gene from the original pTS57 plasmid as well as the sgRNA1/sgRNA3 and *albA* gene sequence from the original JP36_1/JP36_3 and pllk006 plasmids were expected to be both present in pJP1, pJP3 and pJP4 plasmids and were therefore amplified via PCR using primer set TS222/TS223 for *hph* amplification and primer set pllk699/pllk700 for sgRNA amplification with Q5 Hot Start High-Fidelity DNA Polymerase and subsequent 1.5 % Agarose Gel electrophoresis.
The newly assembled plasmids pJP1, pJP3 and pJP4 were then introduced into electrocompetent *A. tumefaciens* EHA105 via electroporation.
Each plasmid was long-time stored at -80 °C by preparing glycerol stocks using 500 µL of a bacterial overnight culture and 500 µL of 50 % glycerol.
The plasmids were again sequenced using the in-house to DIVA DNA Sequencing Service to ensure the correct assembly of fragments before performing further transformation procedures.

2.5. *Agrobacterium tumefaciens*-mediated transformation protocol

Figure 6 presents the workflow for the used ATMT protocol.

T. aurantiacus ascospore preparation was performed by inoculating TEKNOVA PDA plates with WT *T. aurantiacus* cultures and incubating them for 2 days at 50 °C and 4 days at 45 °C. Meanwhile, *A. tumefaciens* strains carrying the right plasmid for transformation were streaked

out on LB-kanamycin plates (50 µg/mL kanamycin) and incubated for two to three days at 30 °C before an *Agrobacterium* colony was randomly picked and transferred into a glass vial with 10 mL liquid LB-kanamycin medium. Incubation was performed overnight at 30 °C on a shaker at 200 rpm.

For the solid and liquid *A. tumefaciens* induction medium preparation containing 200 µM of AS (IM: salts, phosphor buffer, MES-buffer, glucose, thiamine, AS and water), all reagents were sterile filtered with Corning filter systems or small filters and a sterile syringe. Only the agar was autoclaved and added to the prewarmed IM solution. The pH of the IM was adjusted to pH = 5. The IM, especially the AS, had to be prepared freshly on the day of use.

The OD_{600} of the 1:10 diluted bacterial overnight cultures were measured and the cultures were pushed back to an OD_{600} of 0.5 by diluting them with LB-kanamycin medium accordingly. At least duplicates or triplicates were performed per plasmid. After 90 minutes incubation at 30°C and 200 rpm the OD_{600} was re-checked. The samples should be around OD_{600} = 0.85-1 for further processing. Once the cultures reached the expected OD_{600}, they were transferred to 15 mL falcon tubes, spun down for 10 min at 4000 rpm and the supernatant was discarded. Then the pellet was thoroughly resuspended in 10 mL of liquid IM. The centrifugation step was repeated and the pellet was again resuspend in 10 mL IM. The bacterial cells were re-transferred to sterile glass culture tubes, kanamycin was added and the induction was started by leaving the cultures 24 hours on the 30 °C shaker at 200 rpm.

After the *T. aurantiacus* plates were incubated for 6 days and the *A. tumefaciens cultures* have been induced for 24 hours, the actual transformation step was performed. Therefore, the fungal spores were harvested from each plate by adding 5 mL sterile 0.8 % Tween80 solution onto the plate and scraping off the spores with a sterile spatula. This step was repeated twice per plate. The spores were collected in a 50mL falcon tube and all tubes were filled up to the same volume with distilled H_2O. The solutions were filtered through pre-autoclaved Miracloth into a new 50 mL falcon tube to remove unwanted cell material, only the spores flew through Miracloth. The spores were pelleted for 10 min at 4000 rpm and the supernatant was removed until a 10 mL volume in which the pellet was resuspend. A 1:100 dilution was prepared for spore counting using hemacytometer. After counting, the number of spores per milliliter and the total number of spores was calculated in order to dilute the spores to a total of $1*10^8$ spores. The induced *A. tumefaciens* duplicates or triplicates were combined into one 50 mL falcon tube.

For the co-incubation of *A. tumefaciens* cultures and fungal spores a vacuum system consisting of a flask with valve for vacuum and an autoclaved funnel with a sterile cellulose filter was used. Sterile H_2O was used to keep the filter continuously wet. A membrane was placed on top of the filter. 1 mL of fungal spores ($1*10^8$ spores) followed by 2 mL of induced *A. tumefaciens* carrying a certain plasmid were pipetted onto the center of the filter for co-incubation. For each treatment the tweezers had to be changed or sterilized. A negative control was always included by applying only fungal spores onto the membrane which were not transformed with *A. tumefaciens*. The membrane was transferred with a sterile tweezer onto small agar plates containing solid IM (IM plates: salts, phosphor buffer, MES-buffer, glucose, thiamine, AS, water and 1.5 % agar) and the incubation occurred at 28 °C for 48 hours.

After 2 days of co-incubating the fungal spores with *A. tumefaciens*, each membrane was transferred into previously prepared 50 mL falcon tubes containing 250 µg/mL H_2O-cefotaxime solution. Again, for each treatment the tweezers had to be changed or sterilized. The falcon tubes were vortexed until all spores were removed from the membrane and suspended in the 5 mL of H_2O-cefotaxime solution. The blank membranes were removed and the spores were pelleted for 10 minutes at 4000 rpm. The supernatant was discarded carefully until 500 µL in which the fungal spores were re-suspended and streaked out on selection plates. The plates were incubated at 45 °C. After 48 h, the first colonies should appear which could then be further selected and screened for positive transformants.
The same steps were performed for transformation experiments with *A. niger* which served as control organism in this study. A total of $2*10^6$ fungal spores were used for the co-incubation of *A. tumefaciens* with *A. niger*.

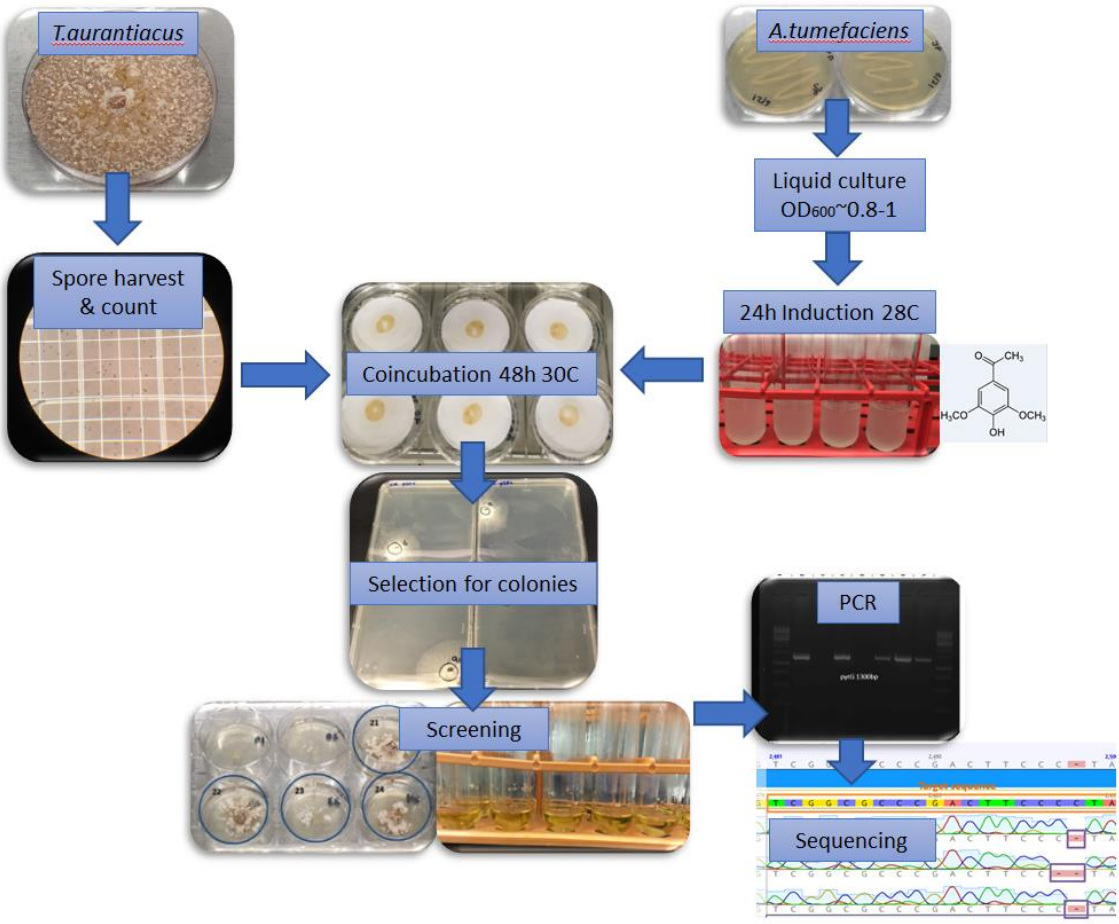

Figure 6: Workflow of previously established ATMT protocol for T. aurantiacus. Cultivation of the T. aurantiacus WT strain was performed on PDA plates for 2 days at 50 °C and 4 days at 45 °C until they produce ascospores which were then harvested and counted. Meanwhile A. tumefaciens strains carrying the right plasmid for transformation were cultured on appropriate selection medium for 2 to 3 days at 30 °C before they were cultured in liquid medium until they reached an OD_{600} of 0.8-1. Then the cultures were induced exactly 24 h at 30 °C on a shaker in IM containing the phenolic compound AS. For the actual transformation $1*10^8$ of fungal ascospores were used for the co-incubation with A. tumefaciens at 28 °C for 48 h. The positive transformants were selected on medium supplemented with hyg B and cefotaxime. Then the transformants were screened for 5-FOA resistance before they were cultured in liquid medium to allow biomass production from which the genomic DNA was extracted. Verification of successfully transformed colonies was performed via PCR through amplification of the

maker gene sequences. Sanger sequencing analysis should finally reveal the desired site-specific mutations in the target gene sequence through sgRNA directed Cas9 cleavage.

2.6. Verification procedures

2.6.1. Selection media

Screening for positive *T. aurantiacus* transformants was performed on different selection media. Hyg B was chosen as the appropriate selection marker to select for transformed fungal colonies with integrated plasmid DNA on PDA media supplemented with 1 g/L uracil, 50 µg/mL hyg B and 200 µg/mL cefotaxime when requested. Cefotaxime was added to the selection medium after transformation in order to eliminate *A. tumefaciens.* Resistant colonies were further selected on Vogel's MM with 2 % sucrose and 1.5 % agar supplemented with 1 g/L uracil and 1 g/L 5-FOA to screen for *pyrG* knockout strains.

Successful *A. niger* transformants were easy to screen for due to the resulting pale conidia of an inactive *albA* allele instead of the characteristic black coloured conidia spores of the *A. niger* WT strain. As selection marker served the *hph* gene responsible for hyg B resistance, thus transformants were selected on Vogel's MM or PDA plates supplemented with 300 µg/mL hyg B and 200 µg/mL cefotaxime after ATMT.

2.6.2. Genomic nucleic acid extraction

Fungal genomic DNA for PCR reactions was isolated from mycelium grown in liquid medium using the Maxwell RSC Plant DNA Kit following the provided protocol and Maxwell RSC Instrument (AS4500). Sample preparation for extraction was performed by adding 0.5 mm Zirconia/Silica beads, one 5.0 mm Yttria bead and part of the fungal mycelium into 2 mL Microtubes with cap and graduation. After adding 500 µL TLA (Tail Lysis Buffer) and 10 µL RNase A to the samples they were processed using MP Biomedicals FastPrep-24™ 5G Instrument for 30 seconds. The sample lysates were centrifuged for 5 min at 15000 rpm to avoid loading any solid particles and the clear lysates were loaded into the Maxwell RSC cartridges for gDNA extraction following the technical manual Maxwell RSC Plant DNA Kit instructions. The concentration of the genomic DNA was determined using NanoDrop2000.

2.6.3. Polymerase chain reaction

All primer sequences used in this study are listed in the appendix. The melting temperature was chosen according to the NEB Tm Calculator (https://tmcalculator.neb.com).

All PCR products were amplified using NEB Q5 Hot Start High-Fidelity DNA Polymerase if not other mentioned. The standard reaction volume was 25 µL including 1X Q5 Reaction Buffer, 0.2 mM dNTPs, 0.5 µM primers, 0.02 U/µL Q5 Hot Start High-Fidelity DNA Polymerase and 0.5 ng of gDNA or plasmid DNA. 50 µL reaction volumes were only performed for subsequent DNA extraction out of the Agarose gel. For fragment amplification for USER Cloning specific primers with an uracil base were used with NEB Phusion U Hot Start DNA Polymerase.

Phusion HS II High fidelity DNA Polymerase was used to generate PCR fragment necessary for pJP1, pJP3 and pJP4 plasmid construction. The colony PCR for assembled JP36_1 and JP36_3 plasmids was performed using LongAmp Taq 2X Master Mix.

Correct PCR amplification was checked via Agarose gel electrophoresis. The agarose percentage was between 0.5 % and 2 % depending on the product length. The gel usually ran at constant 120 V for 30 min.

Table 5: Standard PCR protocol using Q5 Hot Start High-Fidelity DNA Polymerase.

Component	**25 µL reaction**	**50 µL reaction**	**Final concentration**
H2O	16.25 µL	32.5 µL	
5X Q5 Reaction Buffer	5 µL	10 µL	1X
10 mM dNTPs	0.5 µL	1 µL	200 µM
Primer forward (10 µM)	1.25 µL	2.5 µL	0.5 µM
Primer reverse (10 µM)	1.25 µL	2.5 µL	0.5 µM
Plasmid DNA 0.5 ng	0.5 µL	1 µL	<1,000 ng
Q5 Hot Start High-Fidelity DNA Polymerase	0.25 µL	0.5 µL	0.02 U/µL

Table 6: Standard cycling conditions using Q5 Hot Start High-Fidelity DNA Polymerase.

Cycle step	**Temperature**	**Time**	**Nr. of Cycles**
Initial Denaturation	98 °C	30 seconds	1
Denaturation Annealing Extension	98 °C 50 - 72 °C 72 °C	10 seconds 30 seconds 30 seconds per kilobase	30
Final extension	72 °C 4 °C	2 minutes Hold	1

2.6.4. Sequencing analysis

Quick verification of amplified gene sequences was performed through sending purified PCR samples for Sanger sequencing to the external company Genewiz in San Francisco, CA, United States. The purified PCR samples were diluted depending on their product length in a 10 µL volume and premixed with 5 µL of the forward or reverse primer (5 µM) in PCR tubes. The sequencing results were downloaded (.ab1 format) and visualized in Geneious. The ends of the sequences were trimmed ("Annotate & Predict" and "remove trim ends") before they were

aligned to the reference sequence ("Align & Assemble" and "map to reference") in order to locate mutations in the target gene sequence.

Whole plasmid Sequencing Analysis was performed by the In-House DIVA DNA-Seq Service at JBEI. The DIVA DNA-Seq Service is a high-throughput next-generation sequencing service. The Integrative genome viewer (IGV)[31] program was used for visualization of the sequencing data. Both, the reference sequence and the alignment data were imported. Reads are visualized as grey bar, each read has been aligned to a particular place in the reference sequence. On top the coverage track can be seen which is a bar graph that represents the number of reads at a particular location. Important parts are highlighted by a color in IGV which can be mismatches, insertions or deletions. The bolder the color of a base is the higher is the quality and therefore the more likely it is a mismatch. Purple "I"s represent insertions, the number corresponds to the number of inserted bases. Deletions are presented as a black dash.

2.7. Initial optimization strategies

2.7.1. Media testing for cultivation of *T. aurantiacus*

T. aurantiacus WT ascospores were cultured on different media in order to investigate which cultivation medium leads to the most spore production. Therefore, the following cultivation media were tested: Vogel's MM with 2 % sucrose and 1.5 % agar, TEKNOVA PDA and Yeast Extract–Peptone–Dextrose Medium (YPD) medium.

Inoculation was performed in triplicates for each medium and the incubation conditions were 45 °C for six days before the spores were harvested twice in 5 mL sterile H_2O, 1 mL was filtered through a filter tip with sterile miracloth and diluted 1:100 in distilled H_2O. 10 µL of the undiluted or 1:100 diluted spore suspensions were pipetted twice onto the hemocytometer to count the spores. Then the number of spores per milliliter was calculated. The cultivation medium that promotes highest number of spores will be used for culturing *T. aurantiacus* strains for the crossing experiments and for transformation experiments mediated by *A. tumefaciens*.

Ascospores harvested from PDA and Vogel's MM plates were used for ATMT of plasmid pJP1 to compare and investigate whether the used spores have an influence on the transformation efficiency. Positive transformants were selected for hyg B resistance on PDA plates supplemented with 50 µg/mL hyg B, 1 g/L uracil and 200 µg/mL cefotaxime.

2.8. Sexual crossing protocol development

The self-crossing ability of *T. aurantiacus* can be exploited for the establishment of a sexual crossing protocol to generate new fungal strains which harbor two marker genes for easy screening procedures. Therefore, the following mutant *T. aurantiacus* strains were used: taRG008 with hyg B resistance generated through ectopic integration of *hph* via the ATMT procedure as well as the uracil auxotrophic mutant strains 45-1, 45-2, 45-3 and 45-4 which were generated through random UV mutagenesis. We thus expect a new strain that is hyg B as well as 5-FOA resistant and uracil dependent generated through classical sexual crossing.

2.8.1. Isolation of 5-FOA resistant strains

The four *T. aurantiacus* UV mutant strains (45-1, 45-2, 45-3 and 45-4) were generated previously and expected to be 5-FOA resistant and uracil auxotrophic through the integration of random mutations in the genes responsible for 5-FOA resistant auxotrophs, namely in the *pyrG* or *pyrE* gene.

For isolation and verification, the mutant strains as well as the WT strain serving as control, were cultivated for six days on solid PDA plates supplemented with 1 g/L uracil until they produced ascospores which were then used to inoculate liquid medium (Vogel's salts, 2 % mesh milled sorghum, 2 % sucrose and 1 g/L uracil) to allow mycelia growth. After three days, genomic DNA was extracted using Maxwell kit and instrument. To verify that the UV mutant strains have a mutated *pyrG* gene responsible for 5-FOA resistance and uracil auxotrophy, the specific primers LLK683 and LLK686 were designed being complementary to the known genomic target sequence *pyrG* of the *T. aurantiacus* genome and used to amplify this gene region via PCR. For *pyrE* amplification the primer set RG75/RG76 was used. Subsequent Sanger sequencing analysis by Genewiz was performed in order to reveal mutations in one of these genes.

2.8.2. Performance of crossing tests

The mutant *T. aurantiacus* strain taRG008 was chosen to be crossed once with mutant strain 45-1 and once with mutant strain 45-3. All strains were first grown individually on PDA medium supplemented with 1 g/L uracil for two days at 50 °C and four days at 45 °C. For the actual crossing, four quarters were marked on a petri dish with PDA-uracil medium and ascospores from the two strains to be crossed were inoculated twice on opposite facing quarters each. The uracil auxotrophic strains (45-1 and 45-3) were cultured one day earlier than the hyg B resistant strain taRG008. This procedure was performed because of previous observations that the uracil auxotrophic strains germinate slower in comparison to the hyg B resistant strain. That way it could be ensured that the two fungal strains spread equally on the plate. Incubation was at 45 °C for six days.

Once a lawn of ascospores was produced, spore harvest was performed by scraping them off at the interface of the two crossing strains with a sterile spatula (see Figure 7) and transferring them into 750 µL distilled H_2O in a sterile 1.5 mL Eppendorf tube. After vortexing, the spores were filtered through a sterile filter tip with miracloth in it into a new Eppendorf tube. That way the mycelium was retarded and only the spores flew through the filter into the tube. A 1:5 dilution was prepared in order to plate out 100 µL of the diluted spore suspension as well as from the undiluted spore suspension onto big square plates with Vogel's MM (with 2 % sucrose and 1.5 % agar) supplemented with 1 g/L 5-FOA, 1 g/L uracil and 50 µg/mL hyg B in triplicates each to select for successfully transformed strains which gained resistance to both, hyg B and 5-FOA. After incubation at 45 °C for three days the colonies were counted and single colonies were randomly chosen to be sub-cultured on small Vogel's MM plates with 5-FOA, uracil and hyg B for purification reasons in order to get rid of the background and select only for the desired crossed spores. Colonies from each crossing were randomly picked and again sub-cultivated on PDA uracil plates for 6 days before the spore were harvested in distilled H_2O and

cultivated in liquid PD-uracil broth to allow growth of mycelia whereof the genomic DNA was extracted and used for PCR based verification purposes. From all successfully analyzed colonies glycerol stocks were performed to store the crossed strains at -80 °C long-term.

Figure 7: Spore harvest of crossed spores through scraping them off at the interface of the two crossed T. aurantiacus strains from PDA-uracil plates.

To verify that only the generated crossed strain show hyg B and 5-FOA resistance, the following four selection media were prepared in a 24-well-plate format: Vogel's MM, Vogel's MM supplemented with 50 µg/mL hyg B, Vogel's MM supplemented with 1 g/L 5-FOA and uracil each, and Vogel's MM supplemented with 1 g/L 5-FOA, 1 g/L uracil and 50 µg/mL hyg B. Then 5 µL of undiluted filtered spore suspension of the crossed strains as well as the *T. aurantiacus* WT strain and the parent strains serving as controls were cultivated on the four different selection media.

2.8.3. Verification of crossing procedure

For the final proof that the established crossing protocol is reproducible and that the generated strains were pure and can be used for crossings with other mutant *T. aurantiacus* strains, a single colony analysis was performed. A crossed strain was cultivated on PDA medium with uracil for six days at 45 °C. After a lawn of ascospores was produced, the spores were harvested and filtered to retain mycelium. Then the ascospores were counted and a dilution series was performed by diluting the absolute number of spores to reach a total amount of 10^3, 10^2, 10 and 1 spore respectively. From each dilution 500 µL were plated out on Vogel's MM supplemented with 1 g/L 5-FOA, 1 g/L uracil and 50 µg/mL hyg B square plates and the plates were incubated for three days at 45 °C. The aim was to obtain single colonies that could be further analyzed. Single colonies were randomly picked and sub-cultured on PDA medium supplemented with 1 g/L uracil for six days for purification reasons. Then the colony was cultured in liquid PD-uracil (1 g/L) broth for three days at 37 °C to allow biomass production. Genomic DNA was extracted from the mycelium and PCR based screening was performed for verification reasons.

3. Results

3.1. Initial optimization of transformations with ATMT

The established ATMT protocol was used for the first transformation experiments with *T. aurantiacus*. Since very low transformation efficiencies were the results (data not shown), we decided to test different cultivation media in order to investigate what medium leads to the most ascospore production and also which ascospores lead to the highest transformation efficiency. Three different cultivation media will be tested and the two media with the highest spore production will then be used for ATMT in order to find out whether spores from different cultivation media have an influence and can possibly increase the transformation efficiency. The *T. aurantiacus* WT strain was cultured on three different media types, namely PDA, YPD and Vogel's MM. Figure 8 depicts the *T. aurantiacus* WT strain with different growth phenotypes on each of the three tested cultivation media.

Figure 8: T. aurantiacus WT strain phenotypes on PDA, YPD and Vogel's MM from left to right after incubation for six days at 45 °C.

In order to find out what media leads to the most spore production, the number of spores was counted and the absolute spore number was obtained by multiplying the number of spores per milliliter with the total volume of spore suspension. Then the mean and the standard deviation of the triplicates for each used medium was calculated. The results are presented in the bar chart of Figure 9. Cultivation on PDA medium led to the highest ascospore number with a total of $1.5*10^8$ spores whereas $8.6*10^7$ spores were obtained on the Vogel's MM plates. YPD medium lead with $2.7*10^5$ spores to a very low spore production compared to the other two tested media types. Thus, YPD will not be used for further cultivation purposes of *T. aurantiacus*. Based on these results, Vogel's MM and PDA medium were chosen to be used for the ATMT procedure and sexual crossing experiments.

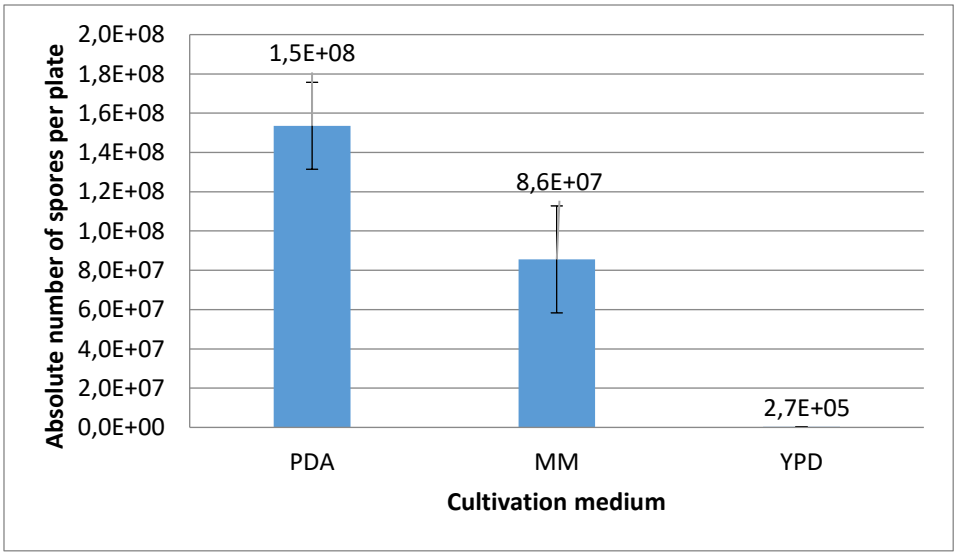

Figure 9: Absolute number of T. aurantiacus ascospores obtained on PDA, Vogel's MM and YPD medium. The values are displayed as mean and standard deviations from three plates each.

The ATMT CRISPR/Cas9 plasmid pJP1 was then transformed into *T. aurantiacus* WT ascospores that were cultivated once on PDA and once on Vogel's MM. Figure 10 presents the resulting number of hyg B resistant transformants obtained through ATMT with ascospores cultivated on the two different media. Transformation of plasmid pJP1 in 10^8 fungal ascospores cultivated on PDA led to about one transformant per plate whereas about 0.5 transformants were obtained using spores cultivated on MM for ATMT through selection on PDA medium supplemented with uracil, hyg B and cefotaxime. Although the transformation efficiency was low in this experiment the tendency of obtaining more positive transformants using *T. aurantiacus* spores grown on PDA plates was observed.

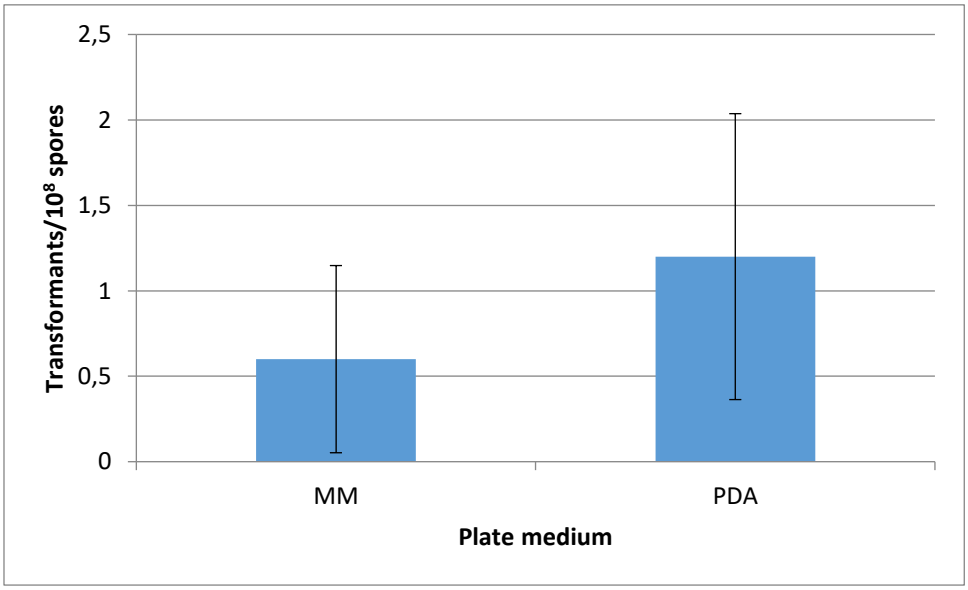

Figure 10: Transformation efficiency for pJP1 plasmid into fungal T. aurantiacus WT ascospores cultivated on Vogel's MM and on PDA. The values are displayed as mean and standard deviations from five plates each.

Since most ascospores were not only counted when the *T. aurantiacus* WT strain was cultivated on PDA medium but also the first transformation experiment demonstrated the trend to get more

transformants using ascospores cultivated on PDA medium, it was decided to use TEKNOVA PDA plates for all further ATMT experiments.

3.2. Development of a sexual crossing protocol

3.2.1. Isolation of 5-FOA resistant strains

For the development of a sexual crossing protocol we needed mutant *T. aurantiacus* strains with different markers in order to combine the gene modifications from different strains and be able to easily select for positive crossed strains. Since there was only a hyg B resistant *T. aurantiacus* strain available and there was no straightforward way of isolating crossed spores at this point, it was decided to generate strains with a second marker. Therefore, strains 45-1, 45-2, 45-3 and 45-4 were generated via random UV mutagenesis and it was then investigated whether they harbor a mutated *pyrG* gene which makes them resistant to 5-FOA and uracil dependent. We were able to amplify the *pyrG* gene sequence for strains 45-1, 45-3 and 45-4 via PCR using specific primers. However, subsequent Sanger sequencing data of the sequenced *pyrG* sequence aligned 100 % to the sequence of the WT strain indicating that no mutations were introduced in the *pyrG* gene region through UV mutagenesis as presented in Figure 12A. Thus, it was assumed that the mutation had to be present in the other gene responsible for 5-FOA resistance, namely *pyrE*.

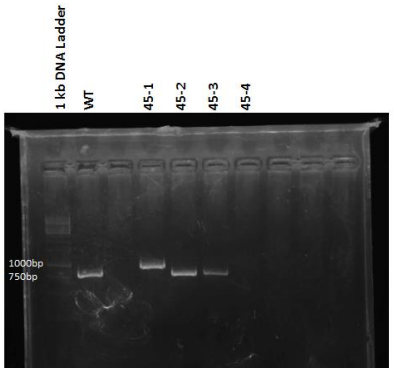

Figure 11: PCR results depict the amplicon of the pyrE gene sequence of T. aurantiacus WT as well as for the UV mutant strains 45-1, 45-2, 45-3 and 45-4.

Figure 11 depicts the PCR results with primer set RG75/76 for the mutant strains 45-1 – 45-4 as well as the *T. aurantiacus* WT control. The obtained product length of the WT was as expected 800 bp. The obtained band for strain 45-1 was at around 1000 bp and hence higher compared to the one of the WT. The obtained bands for strains 45-2 and 45-3 were as big as the one of the WT. For strain 45-4 no band was obtained with the used primer set and this strain was not further investigated. The purified PCR products of the WT, 45-1, 45-2 and 45-3 strains were sent to Genewiz for Sanger sequencing and after aligning the *pyrE* gene sequence of the mutant strains with the WT reference sequence the results revealed that strains 45-1 and 45-3 were mutated in the *pyrE* sequence whereas in strain 45-2 the *pyrE* gene was not deleted because no mutation could be located as shown in figure 12B. In mutant strain 45-1 an insertion of 190 bp was located which turned out to be the duplication of part of the *pyrE* gene sequence and caused a frameshift that inhibits the function of the gene. This finding also correlates with the higher band obtained on the agarose gel (Figure 11). In mutant strain 45-3 a 1 bp insertion

of a Thymine was observed which creates a frameshift mutation disabling the function of the *pyrE* gene, too (see Figure 12C).

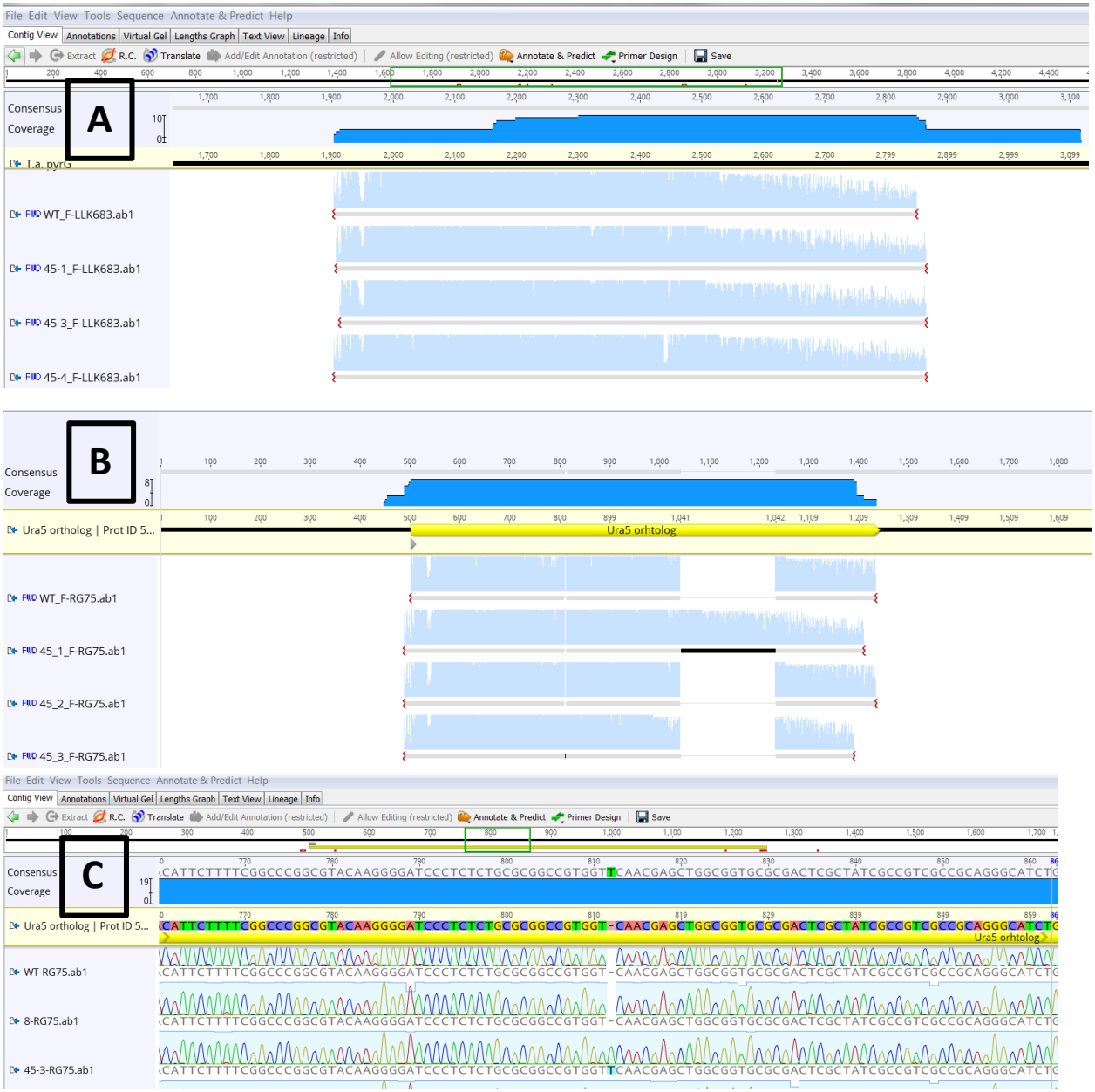

Figure 12: Sanger sequencing data obtained from Genewiz visualized in Geneious. A: T. aurantiacus WT, 45-1, 45-3 and 45-4 strains were aligned to the pyrG reference sequence. B: T. aurantiacus WT, 45-1, 45-2 and 45-3 strains were aligned to the pyrE reference sequence. C: T. aurantiacus strain 45-3 aligned to pyrE reference sequence revealing 1 bp insertion. Pictures obtained from Geneious version 11.1 created by Biomatters.

That way it could be verified that mutant strains 45-1 and 45-3 harbor a deleted *pyrE* gene which makes them uracil auxotrophic and resistant to 5-FOA. This property can now be used as selection marker for simple screening procedures on media with uracil and 5-FOA. That's why it was decided to use these two strains for the following sexual crossing experiments.

3.2.2. Crossing tests

After the successful verification of two *ΔpyrE* strains via PCR and Sanger sequencing, these two strains were chosen to be both sexually crossed with the hyg B resistant strain taRG008.

The crossed strains were then expected to harbor both resistances that could be easily screened for on media supplemented with hyg B, uracil and 5-FOA.

Two sexual crossing experiments were performed with strain taRG008 harboring a *hph* gene cassette once with strain 45-1 and once with strain 45-3. The crossed spores of parent strains 45-3 and taRG008 were selected for hyg B and 5-FOA resistance on Vogel's MM with 1 g/L 5-FOA, 1 g/L uracil and 50 µg/mL hyg B selection plates where a total of 52 colonies were obtained with the undiluted and 16 colonies with the 1:5 diluted spore suspension. 15 colonies (45-3 X taRG008 A-O) were randomly picked for further analysis. From the second crossing (45-1 and taRG008) only one single colony was obtained which was used for further analysis and verification (colony 45-1 X taRG008).

The genomic DNA was extracted from all picked crossed colonies and was used to verify the integration of the *hph* gene cassette responsible for hyg B resistance using TS222/TS223 primers for PCR as well as the deleted *pyrE* gene sequence responsible for 5-FOA resistance and uracil auxotrophy using RG74/RG75 primers for amplification. The purified PCR products were sent to Genewiz to perform Sanger sequencing analysis with the aim to prove that the correct *hph* gene sequences were amplified and to locate the same mutation in the *pyrE* sequence as investigated in the previous isolation experiments from chapter 3.2.1. The agarose gels in Figure 13 depict the expected bands for *hph* gene amplification at 1300 bp for all analyzed crossed colonies as well as in the parent strain taRG008. In *T. aurantiacus* WT strain and mutant strains 45-1 and 45-3 no *hph* gene cassette is integrated, thus no band was expected. Figure 13 shows no bands for these strains. *pyrE* amplicons with a length of about 800 bp were expected for all tested colonies crossed with strain 45-3 whereas a higher band at 1000 bp was expected for the single crossed colony with parent strain 45-1. The PCR amplification was successful for all samples except for colony number J (Figure 13). The *pyrE* amplicon of the 45-1 X taRG008 colony was as expected higher compared to all other tested samples indicating that it contains the 190 bp insertion originating from the parent strain 45-1.

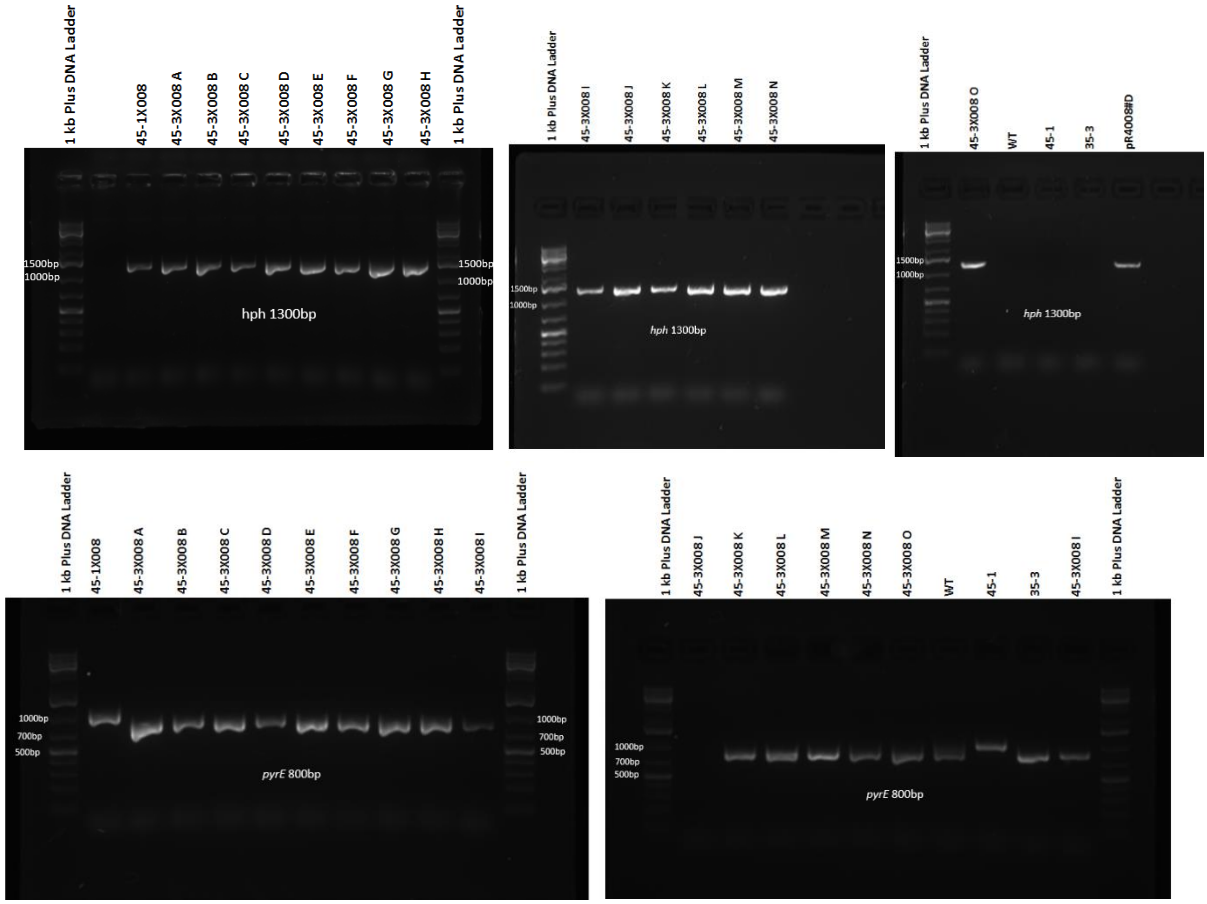

Figure 13: PCR results of crossings 45-1 X taRG008, 45-3 X taRG008 and parent strains taRG008, 45-1 and 45-3 as controls depicting the amplified hph gene cassette on the three agarose gels on the top and the amplified pyrE gene sequence on the two agarose gels on the bottom.

Sanger sequencing results shown in Figure 14 confirm this assumption since parent strain taRG008 had no mutation in the *pyrE* gene sequence but in parent strain 45-1 and in the crossed colony the same insertion could be located. Sequencing data in Figure 15 presents mutations in crossed colonies 45-3 X taRG008 A-F which were located at the same position as the one in parent strain 45-3. Thus, it could be verified that the 1 bp insertion in mutant parent strain 45-3 were present in all analyzed crossed colonies too, whereas this mutation is absent in the control strains taRG008 and *T. aurantiacus* WT.

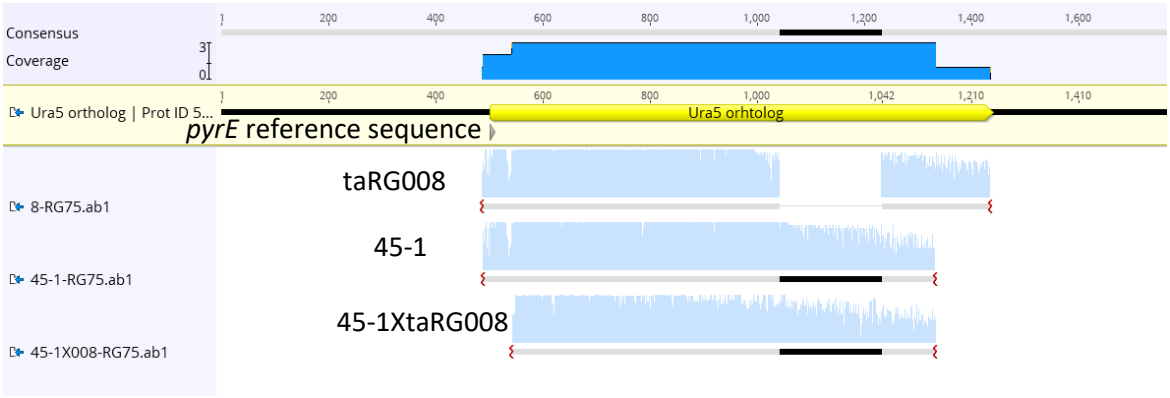

Figure 14: Sanger sequencing data obtained from Genewiz visualized in Geneious. Control taRG008, 45-1 and crossed colony 45-1XtaRG008 was aligned to the pyrE reference sequence. Picture obtained from Geneious version 11.1 created by Biomatters.

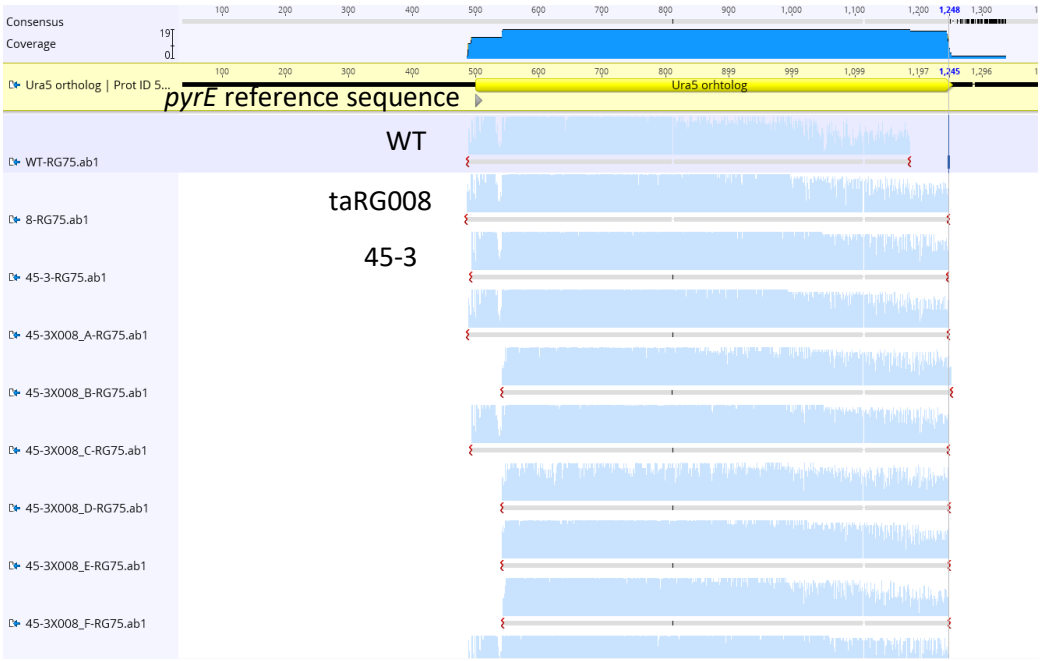

Figure 15: Sanger sequencing data obtained from Genewiz visualized in Geneious. T. aurantiacus WT, control taRG008, 45-1 and crossed colonies 45-3 X taRG008 A-O were aligned to the pyrE reference sequence. Picture obtained from Geneious version 11.1 created by Biomatters.

To conclude, one colony of crossing 45-1 X taRG008 was successfully PCR verified through amplification of the genes *hph* and *pyrE*, sequencing results confirmed the expected 190 bp insertion in the *pyrE* gene. Besides, 15 colonies from crossing 45-3 X taRG008 were analyzed whereof 14 could be successfully verified via PCR and the Sanger sequencing results revealed the expected 1 bp insertion in the *pyrE* gene sequence.

Another way of confirming that the generated crossed strains are the crossing product of strains 45-1 and taRG008 as well as 45-3 and taRG008, was to cultivate them of different selection media. The two crossed strains and the parent strains as well as the *T. aurantiacus* WT strain were cultivated on the four media: Vogel's MM, Vogel's MM supplemented with hyg B, Vogel's MM supplemented with 5-FOA and uracil each and Vogel's MM supplemented with 5-FOA, uracil and hyg B. The WT strain was expected to only grow on MM, whereas strain taRG008 should be also able to grow on media with hyg B. The UV mutants were expected to

show growth only on media supplemented with uracil and 5-FOA due to their uracil auxotrophy. The two crossed strains were expected to grow on all tested selection media.
Figure 16 depicts the 24-well-plate where the two crossed strains and the parent strains as well as the *T. aurantiacus* WT strain were cultivated on the four different selection media. As expected, *T. aurantiacus* WT in the last column (column 6) could only grow on Vogel's MM because it is neither resistant to hyg B nor to 5-FOA. Parent strain taRG008 in column 5 was able to grow on Vogel's MM as well as on MM supplemented with hyg B due to its introduced *hph* gene cassette responsible for hyg B resistance. Mutant strain 45-3 in column 4 showed only growth on media supplemented with 5-FOA and uracil because of its uracil auxotrophy and deleted *pyrE* gene. The strain is resistant to 5-FOA but not to hyg B. Mutant strain 45-1 showed unexpected behaviour because it was supposed to only grow on media with uracil and 5-FOA due to the *pyrE* gene deletion, but in this experiment it was able to grow on Vogel's MM under the absence of 5-FOA and uracil, too.

The crossed strain 45-3 X taRG008 was the only strain that grew on MM supplemented with 5-FOA, uracil and hyg B indicating that this strain had to be a successful crossing product of the parent strains taRG008 and 45-3. However, slower growth was observed on this medium. The strain was also able to grow on all other tested selection media. The second crossed strain, 45-1 X taRG008, was not able to grow on MM supplemented with 5-FOA, hyg B and uracil although one colony could be picked in the previous experiment. Thus, final verification procedures were only performed with the crossed strain 45-3 X taRG008 and it was decided to not use strain 45-1 X taRG008 for further experiments.

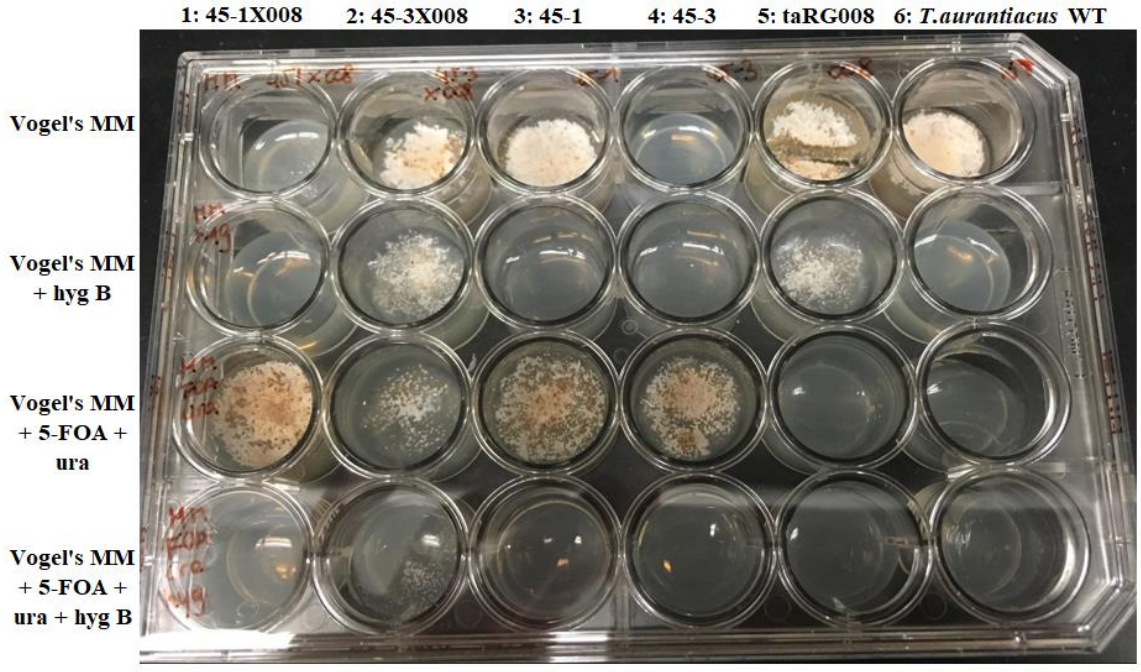

Figure 16: Crossed strains 45-1 X taRG008, 45-3 X taRG008, parent strains 45-1, 45-3, taRG008 and T. aurantiacus WT strain cultivated on the following selection media each: Vogel's MM, Vogel's MM supplemented with hyg B, Vogel's MM supplemented with 5-FOA and uracil each and Vogel's MM supplemented with 5-FOA, uracil and hyg B.

3.2.3. Single spore isolations of the crossed strains

Since no single spore isolation experiments have been performed with the crossed strains yet, the aim was to dilute the spore suspension in serial dilutions in order to obtain single colonies. Those can then be used to verify that the DNA results only from this single colony. For this final verification of the established crossing protocol strain 45-3 X taRG008 B was used. Since UV mutant strain 45-1 grew on MM without uracil, it was decided to use 45-3 strain for further sexual crossing and cloning experiments.

The correct integration of the *hph* gene cassette from the hyg B resistant parent strain as well as the mutation in the *pyrE* gene sequence deriving from the parent strain 45-3 was again verified via PCR and subsequent Sanger sequencing analysis. The results are presented in Figure 17 and Figure 18. The 1.5 % Agarose gel in Figure 17 confirms that the *hph* gene cassette was present in the fungal genome of two randomly picked single colonies of the crossed strain 45-3 X taRG008 B with an expected product length of 1300 bp. A band of the same length was also obtained in the control parent strain taRG008 whereas the band is absent in the second parent strain 45-3 which does not harbor the *hph* gene cassette. Sanger sequencing data in Figure 18 revealed the expected insertion of 1 base (thymine) in the two analyzed crossed colonies which is not present in the *pyrE* reference sequence of *T. aurantiacus* WT but derived from the parent UV mutant strain 45-3. The deleted *pyrE* is responsible for 5-FOA resistance and uracil auxotrophy. Since both marker genes, *hph* and *pyrE*, were amplified in the crossed strain and further verified via sequencing analysis, one can conclude that correct *hph* integration happened and that the resulting strain harbors both marker elements of the parent strains.

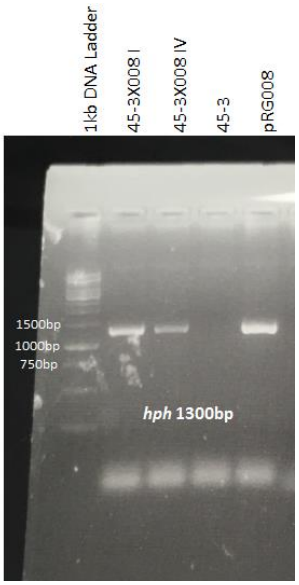

Figure 17: PCR results on a 1.5 % agarose gel depicting hph gene sequence amplicons of two colonies of crossed strain 45-3 X taRG008 next to the parent strains 45-3 and taRG008 as controls.

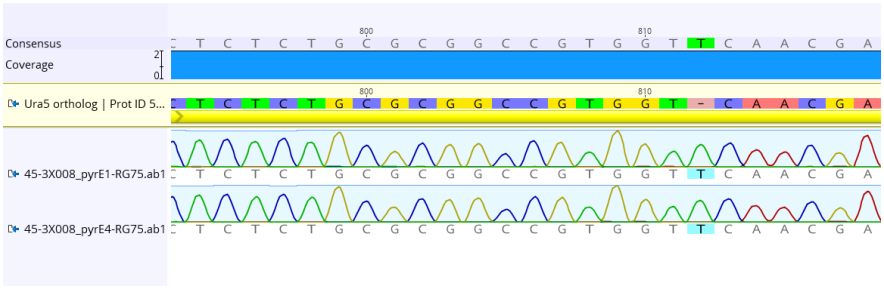

Figure 18: Sanger sequencing data depicts the mutated pyrE gene revealing the 1 bp insertion (Thymine). Sequencing picture obtained from Geneious version 11.1 created by Biomatters.

These results demonstrate that we could reproduce the findings from the previous experiment and that the generated strain 45-3 X taRG008 is a successfully crossing product from the parent strains taRG008 and 45-3. Both, the *hph* gene cassette as well as the deleted *pyrE* gene were confirmed to be present in the fungal genome via PCR-based screening.

3.3. CRISPR/Cas9 plasmid development

The aim was to establish a CRISPR/Cas9 system for the first time for the use with the fungus *T. aurantiacus*. Since previous experiments performed at JBEI indicated that protoplastation was not a successful transformation technique for *T. aurantiacus*, the ATMT protocol was used to randomly integrate plasmids into the fungal genome. For plasmid construction the approach from Nodvig et al[21] was performed in order to generate *A. tumefaciens* plasmids harboring a Cas9 gene and a sgRNA gene targeting the *albA* gene in *A. niger* as a control plasmid and then apply the same strategy to create a CRISPR/Cas9 plasmid and target the *pyrG* gene of the fungal genome of *T. aurantiacus*.

Three different sgRNAs targeting the *pyrG* gene in *T. aurantiacus* were designed and first tested *in vitro* through performing a Cas9 cleavage assay. Figure 19 shows a 2 % agarose gel with the uncleaved control fragment with a length of 1500 bp next to the cleaved *pyrG* sequence that was supposed to be cleaved by Cas9 endonuclease activity directed by sgRNA1, 2 and 3. One can assume from the gel that sgRNA1 directed the Cas9 endonuclease to cleave the whole target sequence into two smaller fragments since two bands (1400 bp and less than 250 bp) appeared on the 2 % agarose gel which are smaller compared to band of the uncleaved control fragment. Using sgRNA2 it seemed that only part of the target sequence was cleaved into two fragments (1000 bp and 400 bp) because a third band still appears at about 1500 bp which is as long as the uncleaved control fragment in lane 2. The experiment with gRNA 3 cleaved the target sequence into two fragments, a band appeared with a length of 700 bp. A very weak band can be seen at the length of the uncleaved fragment, too. Thus, it was decided to use gRNA 1 and gRNA 3 for the following cloning procedures to construct new fungal vectors for Cas9 induced genetic engineering.

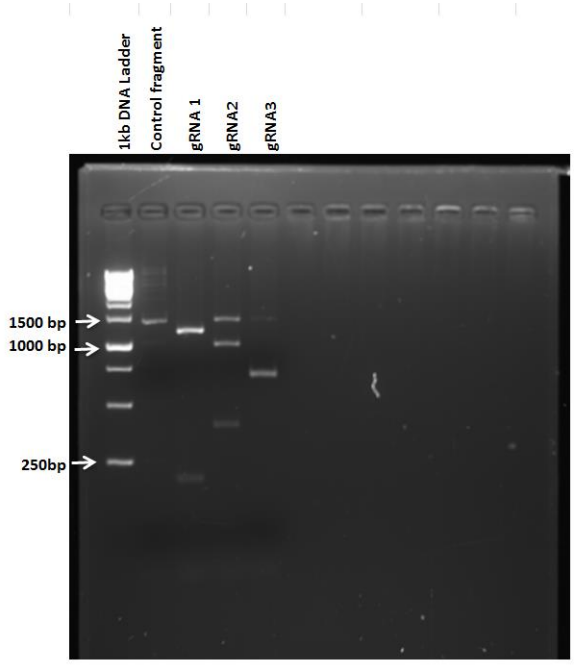

Figure 19: 2 % Agarose Gel depicting 1 kb DNA ladder, the uncleaved control fragment (amplified pyrG gene without Cas9 cleavage) and the Cas9 cleavage of the target pyrG sequence with gRNA 1, 2 and 3.

After performing USER Cloning as described in chapter 2.4.3. the presence of the assembled plasmids JP36_1 and JP36_3 in the cloning host *E. coli* were PCR verified. Figure 20 depicts the PCR amplicons of ten analyzed colonies harboring plasmid JP36_1 with sgRNA1 next to the controls, pllk006 as sgRNA control with an expected product length of 950 bp and pllk003 as *hph* control with an expected product length of 1000 bp. The bands of the colonies were slightly smaller compared to the controls. The same results were obtained for the other assembled plasmid JP36_3 with sgRNA3 (data not shown).

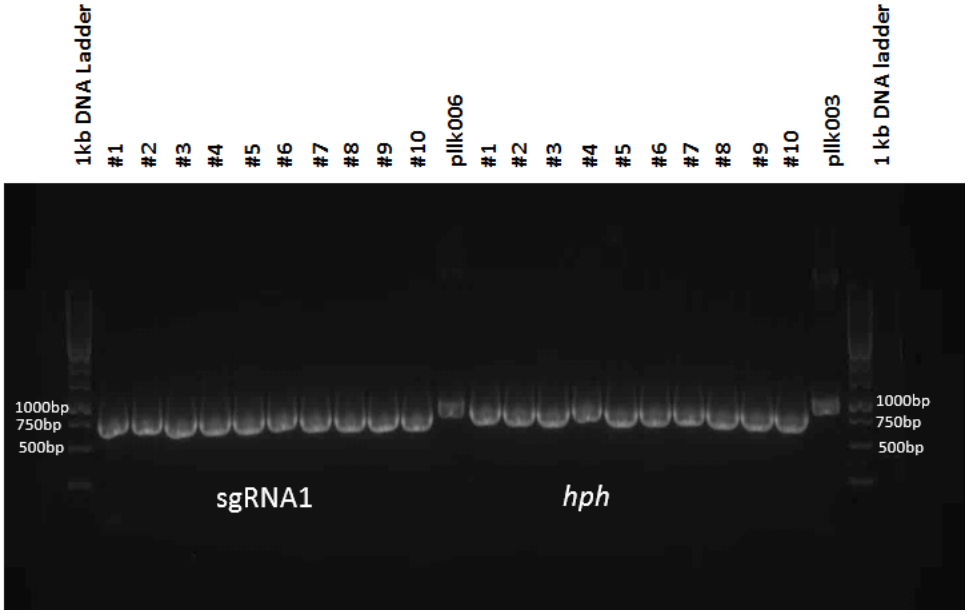

Figure 20: Colony PCR results showing amplicons of the sgRNA gene with an expected length of 950 bp (left) and the hph gene with an expected product length of 1000 bp (right) of 10 analyzed E. coli colonies harboring plasmid JP36_1.

The purified PCR products of colonies#1-5 were sequenced. Sanger sequencing analysis performed by Genewiz revealed that the correct fragments were amplified because the sequence data aligned to the reference sequence. Besides, each analyzed colony harbored the introduced protospacer sequence as well as the desired reverse complement of the protospacer sequence as highlighted in Figure 21. This indicates that USER Cloning lead to the correct assembly of the two vectors pllk003 and pllk006.

Figure 21: Sanger sequencing analysis of five E. coli colonies harboring plasmid JP36_1. The protospacer sequence for sgRNA 1 and the reverse complement sequence of the protospacer are highlighted. Picture obtained from Geneious version 11.1 created by Biomatters.

Figure 22 presents the sequencing data of the whole plasmid obtained from DIVA. The figure shows the relevant gene cassette from the *gdpA* Promoter to the *Tef1* terminator which was later introduced into the *A. tumefaciens* vector pTS57. Again, it could be confirmed that both plasmids harbor the correct protospacer sequence. Besides, in every colony an insertion of 5 bases CTCAG presented as a purple "I" was observed at the end of the *trpC* terminator region in all reads as well as two mismatches in the Cas9 region (T instead of C) which are demonstrated as a red "I" in the IGV software can be observed from the data. The two mismatches in the Cas9 gene sequence do not lead to a frameshift and therefore do not change the encoded amino acid. Thus, the mismatches were tolerated and since no other mismatches were observed in all reads of the sequencing data it can be concluded that the designed plasmids were proven to be error free and can be used for Gibson Assembly to integrate the sgRNA-Cas9 gene cassette from JP36_1/JP36_3 into the *A. tumefaciens* vector pTS57 necessary for ATMT.

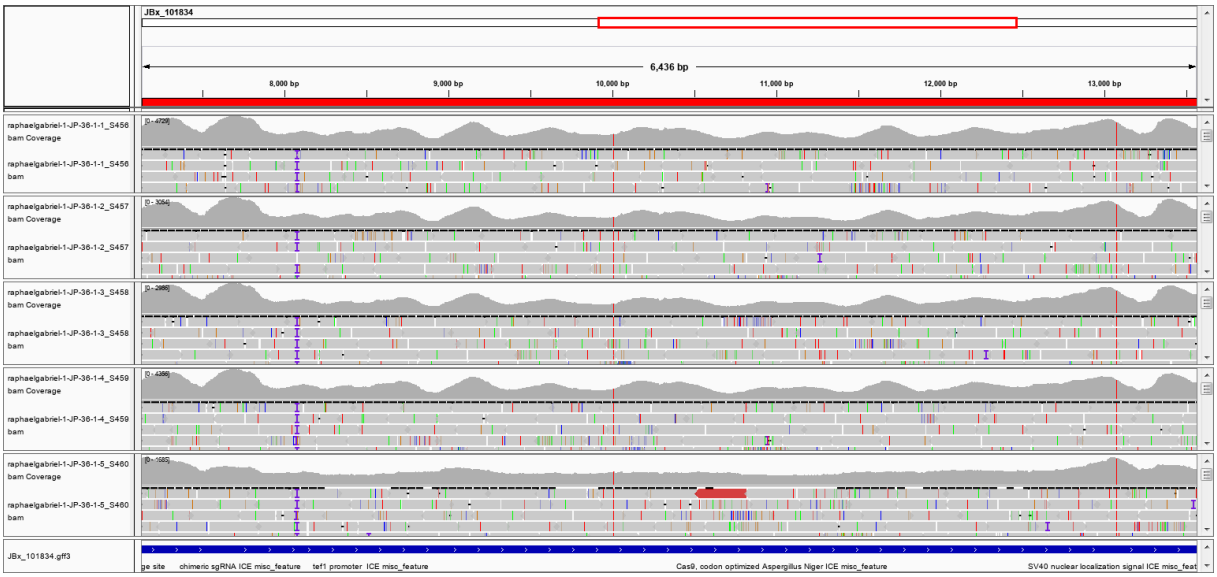

Figure 22: Whole Plasmid Sequencing data of JP36_1 colony#1-5 showing the gene cassette from the gdpA promoter to the Tef1 terminator visualized in IGV.

After assembling the plasmid pJP1, deriving from JP36_1 and pTS57, and pJP3, deriving from JP36_3 and pTS57, and transforming them into *E. coli*, PCR based verification was performed again. The sgRNA gene sequence from the original JP36_1/JP36_3 plasmids as well as the *hph* gene from the original pTS57 plasmid were expected to be present in the newly assembled plasmids. The expected results were obtained and correct assembly of the two vectors via Gibson Assembly could be confirmed since a sgRNA band was obtained at the expected length of 950 bp and a band for *hph* integration was obtained at an expected length of 1000 bp for three randomly picked colonies (agarose gels not shown). DIVA sequencing data revealed the same mismatches as already observed in plasmid JP36_1 and JP36_3 indicating that they derive from the original plasmids used for USER Cloning and did not occur during the performed cloning procedure.

Also plasmid pJP4, deriving from pllk006 and pTS57, was assembled through Gibson Assembly and the integration of the correct sgRNA sequence was successfully verified via PCR followed by Sequencing analysis through Sanger sequencing and whole plasmid sequencing by DIVA (data not shown).

All three constructed CRISPR/Cas9 plasmids, pJP1, pJP3 and PJP4, were confirmed to harbor the desired protospacer and reverse complement sequence and the DNA contained no errors and thus used for electroporation into *A. tumefaciens* to use them for transformation of fungal *T. aurantiacus* WT ascospores via the ATMT protocol.

3.3.1. Expression of CRISPR/Cas9 through ATMT in *A. niger* for deletion of the *albA* gene

Research work performed by Nodvig et al[21] demonstrates the successful deletion of the *albA* gene in *A. niger* using the CRISPR/Cas9 genetic engineering system. Thus, it was decided to use the same sgRNA and apply the same cloning strategy and transform the plasmid with ATMT into the fungal genome to delete the *albA* gene in *A. niger*. Hence, the functionality of the designed CRISPR/Cas9 systems with ATMT was firstly tested in filamentous fungus *A.*

niger which is a closely related filamentous fungus to *T. aurantiacus* and served as positive control in this study. Therefore, vector pJP4 constructed through Gibson Assembly of pllk006 and pTS57 harboring a sgRNA gene cassette with a protospacer targeting the phenotypic marker gene *albA* of *A. niger* was introduced into the fungal genome through ATMT as described in chapter 2.5.

Figure 23 shows *A. niger* colonies grown on PDA plates supplemented with 300 µg/mL hyg B and 200 µg/mL cefotaxime incubated at 30 °C. Potential positive *A. niger* transformants firstly developed a white phenotype, after further incubation for a few days the expected pale conidia were observed (colonies circled in blue in Figure 23) which imply that the Cas9 nuclease was guided to induce mutations and thus delete the function of the *albA* gene. In total, eight potential *albA* transformants were randomly picked and sub-cultivated on PDA plates supplemented with 300 µg/mL hyg B plates at 30 °C for purification reasons for 6 days at 30 °C (Isolates A-H). Five out of the eight isolates developed again sectors with pale conidia as presented in Figure 24 on the left indicating a deleted *albA* gene, whereas on the three isolates on the right of Figure 24 hardly any pale sector or no pale candida could be observed. Instead, they showed the same phenotype as the *A. niger* control with the characteristic black conidia spores indicating that these colonies were not successfully transformed. Thus, the five potential *ΔalbA* colonies were chosen to be further analyzed.

Figure 23: A. niger colonies on PDA-hyg B plates with potential albA knockout are circled in blue.

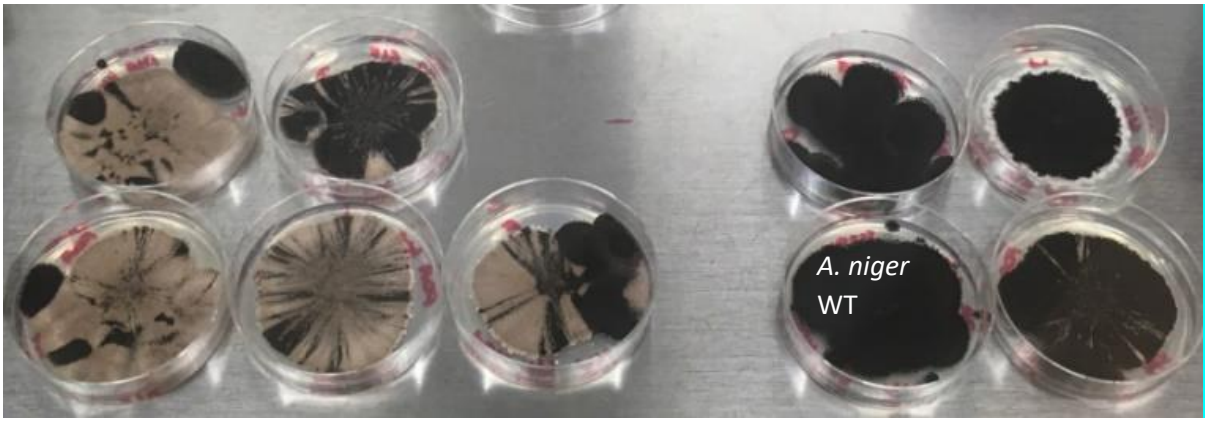

Figure 24: Eight A. niger isolates and A. niger WT sub-cultured on PDA-hyg B medium for six days at 30 °C.

In order to confirm that the pale phenotype is the result from causative mutations in the *albA* gene that was induced by the specific albA-sgRNA-Cas9 nuclease, part of the pale sectors of the purified colonies were used for inoculation of liquid PD broth. After DNA extraction the genomic DNA was used to generate PCR fragments of the region in the *albA* gene that was targeted by Cas9 with a specific primer set. A band was expected at 1700 bp. The agarose gel in Figure 25 confirmed the expectation because for all five isolates a band was obtained with the correct length.

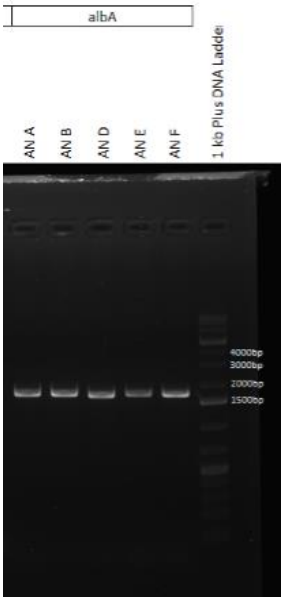

Figure 25: PCR results on a 1 % Agarose Gel depict albA amplicons of five transformed A. niger colonies using LongAmp Taq Polymerase at an expected product length of 1700 bp.

The PCR amplicons were Sanger sequenced and aligned to the *albA* reference sequence in the Geneious software to locate mutations of the potential *A. niger* transformants. In Figure 26 the target sequence was highlighted in orange. This is the part in the *albA* gene that base-pairs with the designed protospacer sequence in the sgRNA region of vector pJP4 (AGTGGGATCTCAAGAACTAC) and within this protospacer sequence DSBs were expected to be introduced three nucleotides upstream of the PAM sequence. In this case the PAM is TGG which is highlighted in red in the target *albA* sequence. In all five analyzed cases mutations were identified at the expected site to be cleaved by the endonuclease. The mutation was either an insertion, deletion or a mismatch of one of more bases. All mutations were located three base pairs upstream of the PAM sequence. Colony number A revealed four random mismatches and one insertion in the target sequence. Also in colony number B several mismatches were observed but with an insertion of four bases. In colony D a 44 bp deletion was introduced through the applied CRISPR/Cas9 system. In colonies E and F exactly the same mutations were observed, in both cases a 6 bp deletion occurred during DNA repair. The results demonstrate that these mutations were most likely obtained by error-prone NHEJ repair of the DSBs that were induced by Cas9.

Since five out of eight inoculated colonies developed again pale sectors after sub culturing and all five colonies were verified to have a deleted *albA* gene a transformation efficiency of 62.5 % can be concluded from this experiment.

Thus, it was confirmed that the basic components of the used CRISPR-Cas9 system worked in *A. niger* and therefore it was further investigated whether specific CRISPR-Cas9 vectors can also be used for ATMT in the closely related filamentous fungus *T. aurantiacus*.

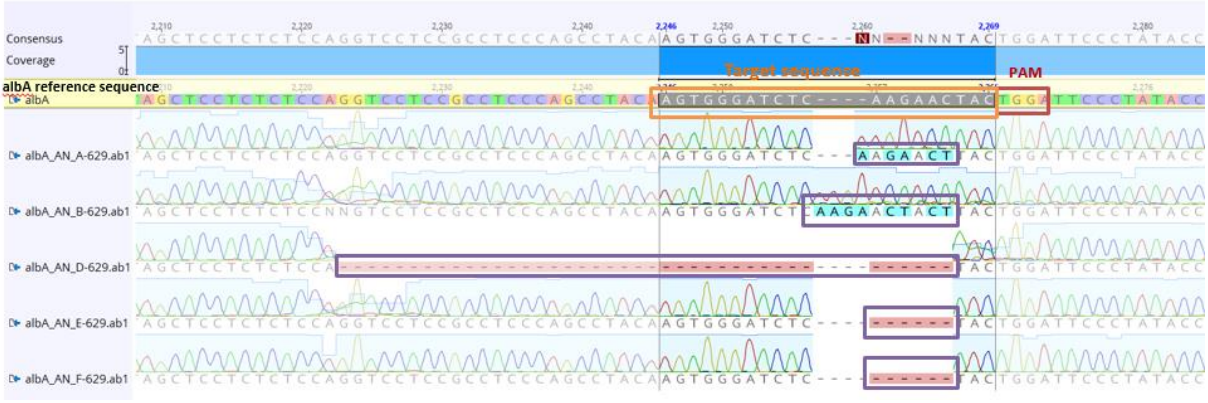

Figure 26: Sanger sequencing results for five analyzed A. niger transformants revealing mismatches, insertions and/or deletions framed in purple through Cas9 cleavage three nucleotides upstream of the PAM sequence (framed in red) in the albA target sequence (framed in orange). Picture obtained from Geneious version 11.1 created by Biomatters.

3.3.2. Expression of CRISPR/Cas9 through ATMT in *T. aurantiacus* for deletion of the *pyrG* gene

After successful introduction of the CRISPR/Cas9 vector via ATMT into the control organism *A. niger*, the same strategy was used to randomly integrate the two designed CRISPR/Cas9 vectors pJP1 and pJP3 into the fungal genome of *T. aurantiacus* and introduce site specific DSB in the *pyrG* gene upon expression of the sgRNA and Cas9 genes. As a proof of principle, the *pyrG* gene was chosen as a target locus for editing. Therefore, two expression plasmids were constructed only differing in their protospacer sequence and reverse complement sequence of the protospacer, both targeting the *pyrG* gene region in *T. aurantiacus*. The plasmids contain a *PgpdA*-sgRNA-*TtrpC* and *Ptef1*-Cas9-*Ttef1* cassette as well as the *Ptef1-hph-TtrpC* cassette used as selection marker to screen for hyg B resistance. Introduction of the plasmids in the WT strain *T. aurantiacus* was performed via the described ATMT procedure.

Plasmid pJP1 was transformed into *T. aurantiacus* in two individual experiments. For all attempts the spores were counted and diluted to use a total of $1*10^8$ fungal spores for co-incubation with 2 mL of induced *A. tumefaciens* cultures carrying the right plasmid to be inserted into the fungal genome. For the first experiment, fungal spores grown on Vogel's MM and grown on PDA were tested as already reported in the initial optimization strategy chapter 3.1. The used AS concentration was 300 µM. Three potential positive transformants were obtained through transformation with fungal spores grown on Vogel's MM whereas six colonies were obtained through transformation with spores cultivated on PDA medium. In total, nine potential positive transformants were observed on ten PDA selection plates supplemented with 1 g/L uracil, 50 µg/mL hyg B and 200 µg/mL cefotaxime. For the negative control where *T. aurantiacus* was not transformed with *A. tumefaciens* no colonies were obtained on the selection plate. Since the transformation efficiency appeared to be higher with *T. aurantiacus* spores that are cultivated on PDA, this type of medium was used for the further transformation rounds.

All positive transformants were sub-cultivated for purification reasons before they were further selected on Vogel's MM as well as on Vogel's MM supplemented with 1 g/L 5-FOA and uracil each to investigate whether the *pyrG* gene was knocked out and therefore the fungus gained resistance to 5-FOA. Two controls were also included, *T. aurantiacus* WT served as control which was only able to grow on Vogel's MM whereas the *ΔpyrG* control strain 45-2 was only able to grow on media with 5-FOA and uracil. All potential transformants were able to grow on Vogel's MM, but one out of the nine transformants (transformant E) showed additionally resistance to 5-FOA indicating that the CRISPR/Cas9 system performed gene editing in the *pyrG* gene sequence in the genome of *T. aurantiacus*. This colony was isolated and cultured in liquid PD broth with uracil (1 g/L) in a 24-well-plate in order to extract genomic DNA out of the mycelium and perform a PCR for verification of the integration of the plasmid into the fungal genome and the site specific cleavage of the *pyrG* gene that was induced by the specific *pyrG*-sgRNA-Cas9 nuclease activity. The mutation in this target gene was verified by Sanger sequencing. The resulting data revealed that in the *pyrG* gene sequence of transformant E a mutation was introduced in the protospacer sequence site supposed to be cleaved by Cas9. Figure 27 displays the Sanger sequencing results of transformant E aligned to the *pyrG* reference sequence. A large 64 bp random insertion (framed in purple) was located four base pairs upstream of the PAM site (framed in red). This data demonstrates that the CRISPR/Cas9 system was able to mutate the target gene *pyrG* in the *T. aurantiacus* genome through DSB repair that was mediated most likely by NHEJ events and lead to a frameshift in the gene. Since in one out of nine potential *T. aurantiacus* transformants a site-specific mutation was located and introduced through the applied CRISPR/Cas9 technology, a transformation efficiency of 11 % was achieved in this very first transformation experiment using the CRISPR/Cas9 plasmid pJP1 and the ATMT procedure.

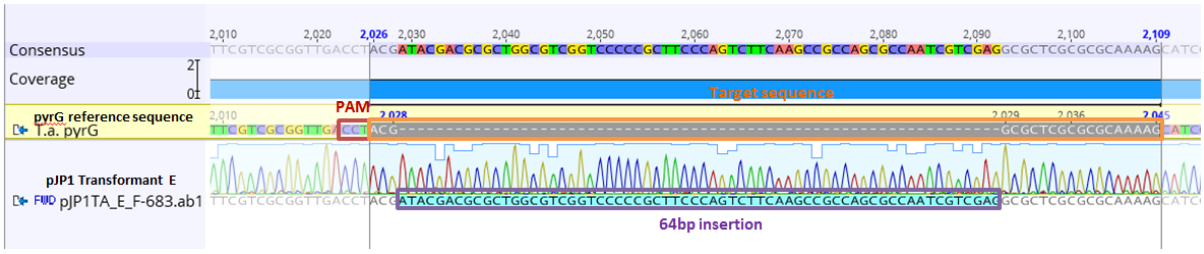

Figure 27: Sanger sequencing results for T. aurantiacus pJP1 transformant E revealing a 64 bp insertion framed in purple through Cas9 cleavage three nucleotides downstream of the PAM sequence (framed in red) in the pyrG target sequence (framed in orange). Picture obtained from Geneious version 11.1 created by Biomatters.

In order to confirm the successful gene editing results through the CRISPR/Cas9 system and ATMT described above and to evaluate the reproducibility of the transformation potential in *T. aurantiacus* the experiment was repeated with vector pJP1. Besides, this time also plasmid pJP3 plasmid was transformed, which differs only in the 20 bp protospacer and 6 bp sequence in the HH region of the sgRNA gene from the sequence of pJP1. Both sgRNAs target the *pyrG* gene. The following alterations were performed expecting to obtain higher transformation efficiencies. All reagents used for transformation were prepared freshly and the pH of the IM was measured and adjusted to pH = 5.0 as expecting it to be a critical parameter during the ATMT procedure. The AS concentration was adapted to 200 µM.

After ATMT, the selection for positive transformants was performed through screening for hyg B resistance first. That is why the transformed fungal spores were selected again on PDA plates supplemented with uracil, hyg B and cefotaxime. After two days incubation at 45 °C, 95 transformed colonies were obtained with an integrated pJP1 plasmids whereas 60 colonies were counted with the inserted plasmid pJP3. Thus, on average five transformants were obtained per plate harboring pJP1 plasmid and three transformants were the result of transformation with pJP3 plasmid as presented in Figure 28.

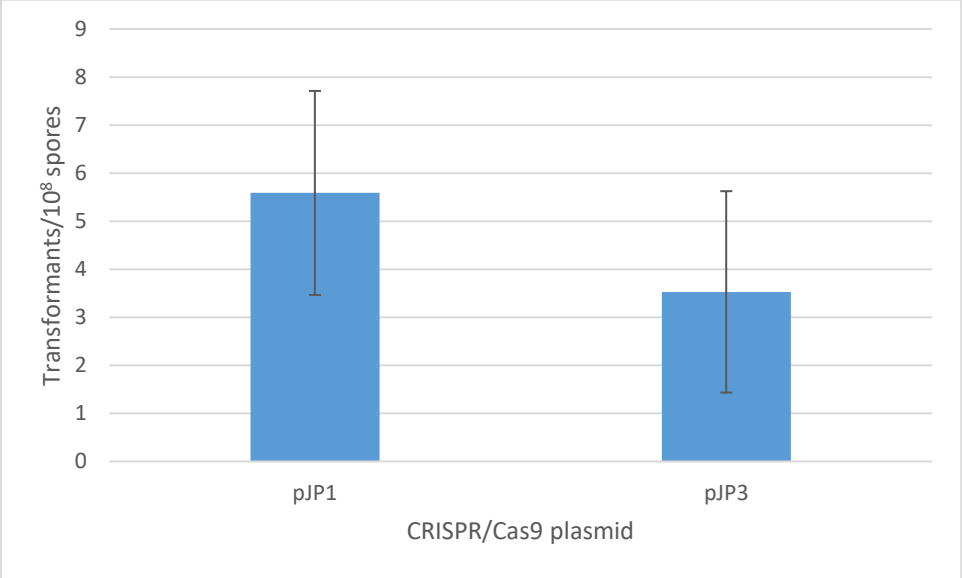

Figure 28: Transformation efficiency for pJP1 and pJP3 transformants selected on hyg B plates. The values are displayed as mean and standard deviations from 17 plates each. $1*10^8$ T. aurantiacus ascospores were used for the transformation process.

A subset of 20 potentially transformed *T. aurantiacus* colonies harboring pJP1 and 20 potential *T. aurantiacus* transformants harboring pJP3 were randomly picked from the hyg B selection plates and were further screened for 5-FOA resistance on Vogel's MM supplemented with 5-FOA and uracil in 6-well-plates, this time without another sub-cultivation step. *T. aurantiacus* WT and UV mutant 45-3 served as controls for this experiment. That way we wanted to test whether we can select the transformants immediately for 5-FOA resistance without a previous sub cultivation step which would shorten the whole screening process for one week of incubation time. Incubation was performed at 45 °C for six days.

Figure 29 presents the screening results of the transformations on 5-FOA selection plates. Three out of 20 transformed *T. aurantiacus* colonies (colony number 11, 12 and 16) harboring pJP1 plasmid showed 5-FOA resistance whereas 12 out of 20 transformed colonies (colony number 21-24, 28-30, 34-37) with pJP3 plasmid were able to grow on media containing 5-FOA. It was observed that the transformants with pJP1 grew slightly slower compared to transformants harboring pJP3 plasmid. The WT control did not grow on 5-FOA, but *ΔpyrG* control strain 45-3 showed as expected growth on the selection medium. All named 5-FOA resistant colonies were cultivated in liquid minimal medium containing Vogel's salts, soy peptone and uracil to extract genomic DNA out of the mycelium and perform PCR based verification of a *pyrG* gene editing through the established CRISPR/Cas9 technology.

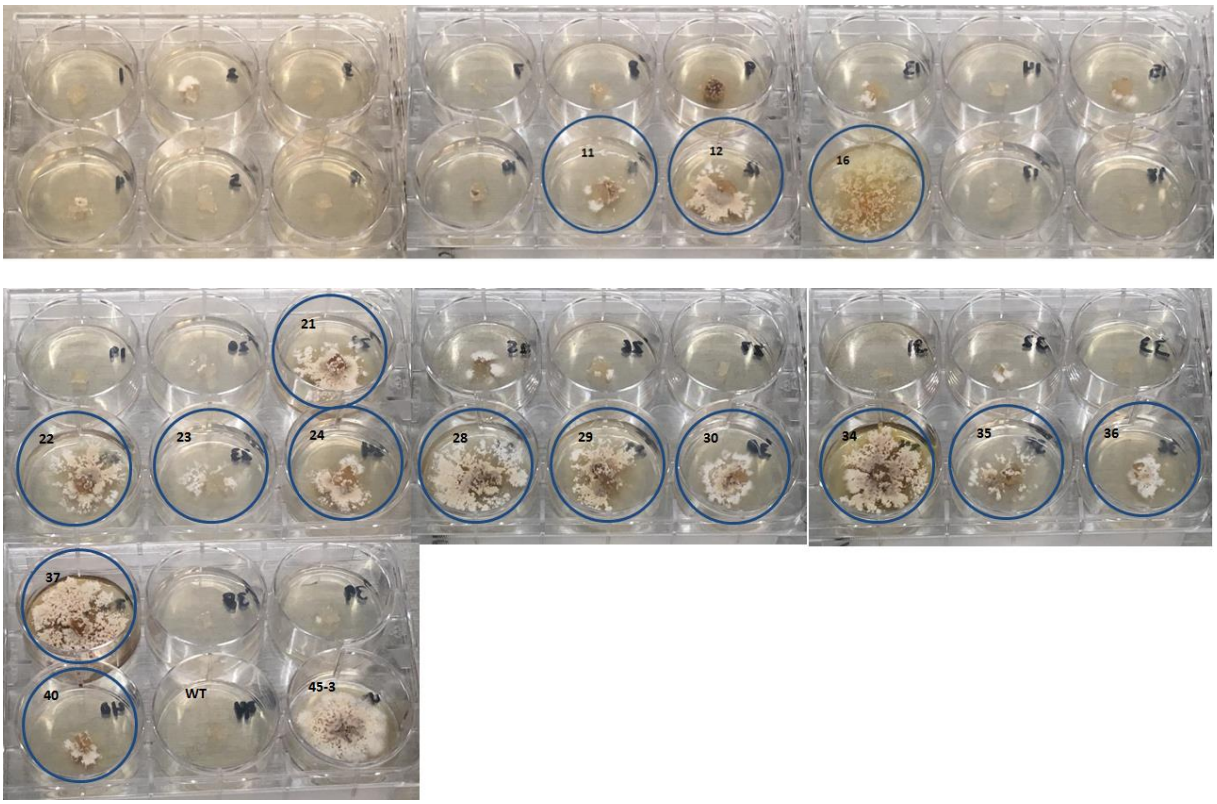

Figure 29: A subset of 20 colonies harboring pJP1 and pJP3 were selected for 5-FOA resistance each. pJP1 colony 11, 12 and 16 as well as pJP3 colony 21, 22, 23, 24, 28, 29, 30, 34, 35, 36, 37 are positive transformants on the selection medium and where used for verification procedures.

Subsequent Sanger sequencing analysis revealed that in two of the three potential transformants with pJP1 plasmid a *pyrG* knockout could be confirmed. In colony#12 a large insertion of 94 bp was observed in the target gene of *T. aurantiacus* that was introduced four base pairs upstream of the PAM sequence. In colony number 16 the CRISPR/Cas9 system led to a deletion of three base pairs. In the third analyzed colony (#11) no site specific mutation was located on the target site supposed to be cleaved by sgRNA1 directed Cas9 endonuclease. Hence, we achieved again a transformation efficiency of 10 % with the CRISPR/Cas9 plasmid pJP1 targeting *pyrG* in *T. aurantiacus*.

From the 12 transformed colonies harboring plasmid pJP3 the deleted *pyrG* gene could be confirmed in seven colonies via PCR and Sanger sequencing analysis. In all colonies exactly the same base was deleted 3 bp upstream of the PAM site creating a frameshift. In colonies number 21, 22, 23, 34 and 37 only this 1 bp deletion of a cytosine was revealed whereas in colony#28 another cytosine base was additionally deleted and in colony#29 three additional mismatches were found. For the other transformed colonies a *pyrG* knockout could not be verified because either it was not possible to amplify the gene sequence with the used primer set or due to insufficient quality of the sequencing data. Consequently, we reached a transformation efficiency of 35 % with plasmid pJP3 which is higher compared to the one achieved with pJP1.

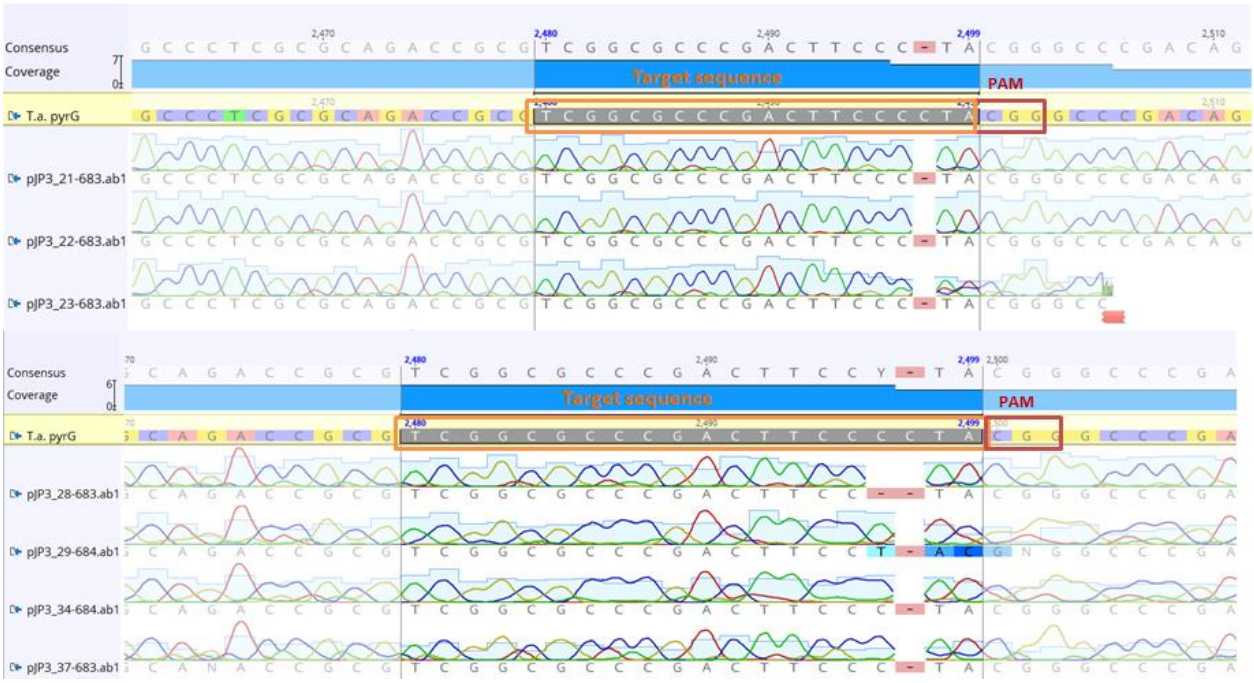

Figure 30: Sanger sequencing results for T. aurantiacus pJP3 transformants revealing deletions and mismatches through Cas9 cleavage three nucleotides downstream of the PAM sequence (framed in red) in the pyrG target sequence (framed in orange). Picture obtained from Geneious version 11.1 created by Biomatters.

The transformation efficiencies obtained from all performed transformation experiments, namely ATMT of *A. niger* targeting the *albA* gene as well as both transformation experiments of *T. aurantiacus* targeting the *pyrG* gene are summarized in Table 7. In principle, the efficiency for *T. aurantiacus* was much lower compared to the one for *A. niger*. Interestingly, transformation with pJP1 plasmid led to more hyg B resistant colonies but the pJP3 transformed colonies seemed to grow faster and more of those analyzed transformants had an expected mutation in the *pyrG* gene.

Table 7: Transformation efficiencies with CRISPR/Cas9 plasmids pJP4 targeting albA in A. niger and pJP1 and pJP3 targeting pyrG in T. aurantiacus.

ATMT round	**Introduced CRISPR/Cas9 plasmid**	**No. of filters**	**No. of hyg B resistant colonies**	**No. of further analyzed colonies**	**No. of 5-FOA resistant colonies**	**No. of *ΔalbA/ ΔpyrG* colonies**	**Efficiency**
1	pJP4	3	22	8	/	5	62 %
2	pJP1	10	9	9	1	1	11 %
3	pJP1	17	95	20	3	2	10 %
3	pJP3	17	60	20	12	7	35 %

4. Discussion

In this study, a strain engineering technique was successfully established which is able to delete single genes in the tested fungal species *A. niger* and *T. aurantiacus* based on CRISPR/Cas9. We randomly introduced the Cas9-encoding gene into the genome of *A. niger* and *T. aurantiacus* via the *ATMT* procedure. Over the last few decades, scientists have already been trying to establish new genetic tools to improve and facilitate genetic engineering methods in filamentous fungi[20]. With the powerful CRISPR/Cas9 system a lot of time can be saved to generate desired mutant strains with different deleted genes simply by changing the 20 bp long protospacer sequence of the sgRNA as well as the 6 bp reverse complement sequence to the target locus according to the fungal genomic sequence. Although it still takes about one month to fully verify the correct expression of Cas9 in *T. aurantiacus*, the process for generating genome-wide gene deletions in filamentous fungi is shortened using the CRISPR/Cas9 technology.

Cas9 has the potential to significantly enhance gene modifications in *T. aurantiacus* as in other tested fungi. During this study three CRISPR/Cas9 vectors were constructed via USER Cloning and/ or Gibson Assembly and re-sequenced to ensure that the DNA contained no error. Two of them harbor an sgRNA targeting the auxotrophic *pyrG* gene in *T. aurantiacus* and the third vector carries a sgRNA targeting the phenotypic gene *albA* in the control organism *A. niger*. With the use of this efficient CRISPR/Cas9 system, the marker gene *pyrG* gene was knocked out to create mutant *T. aurantiacus* strains that can be selected on 5-FOA and uracil plates which can be used as an easy screening method in the future. In two individual experiments a Cas9 targeting efficiency of 10-11 % was obtained using sgRNA1 whereas an even higher efficiency of 35 % was achieved using the other guide RNA (sgRNA3) targeting *pyrG*. Selection for positive transformants was first performed on hyg B plates and then the colonies were further screened for 5-FOA resistance indicating a successfully deleted *pyrG* gene. It was observed that the Cas9 endonuclease was not 100 % efficient and the reason for the lower transformation efficiency with sgRNA1 might be due to unknown off-target effects of other genes. Thus, the choice of the sgRNA seems to be essential for the resulting transformation efficiency.

Deletion of the *pyrG* gene enables us now to use this gene as a recyclable marker and expand the repertoire of selection strategies for transformants. This gene has also been used as target for CRISPR/Cas9 in model fungi like *A. niger*[21]. Still, it has to be further tested whether *pyrG* knockout strains can be directly selected on 5-FOA medium without selecting transformed colonies first on hyg B plates to use the *pyrG* as a direct selection marker as well as to shorten the time for the verification procedures. Besides, the same CRISPR/Cas9 system was also applied for the closely related fungus *A. niger* where a transformation efficiency of 62.5 % was the result. Studies using the CRISPR/Cas9 technology have already been performed to knock out different genes in *A. niger* with disruption rates of up to 97 % indicating that efficient genome editing is possible[32].

The CRISPR/Cas9 system was established for genome editing for the first time in the thermophilic species *T. aurantiacus* and has the potential to accelerate genome-wide metabolic engineering of this fungus to produce cellulases. The obtained results in this study confirmed

that the ATMT CRISPR/Cas9 technology was successfully editing the genome of two filamentous fungi with precision and efficiency, similarly to other organisms.

Furthermore, during this research work, a classical sexual crossing protocol was successfully established for the fungus *T. aurantiacus.* We exploited the self-fertilizing capability of this fungal species to easily and quickly create a new mutant strain with two resistances, in this case we generated two strains with both hyg B resistance as well as 5-FOA resistance and uracil auxotrophy. An advantage of the homothallic characteristic of *T. aurantiacus* is that the obtained crossed spores are all purified and genetically identical. The performed experiments confirmed that selection for crossed colonies was possible on medium containing hyg B, 5-FOA and uracil even though slower growth was observed possibly due to the high selection pressure. Crossed colonies of parent strains 45-3 and taRG008 were verified in independent experiments which confirmed that the established sexual crossing protocol works and can be used for stacking mutations in *T. aurantiacus* within a short period of time.

Although the development of genetic tools greatly simplifies the introduction of site-specific gene modifications in the genome of *T. aurantiacus,* it is also important to establish bioprocess engineering strategies in order to get insight into how cellulase can be induced by sugars and also to assess the optimal bioreactor parameters for industrial scale enzyme production[9]. This is necessary to achieve increased yield and enable cost-efficient biomass conversion to biofuels and bio-products[33]. Previous studies have been performed to investigate what sugars can be used to induce the cellulase and xylanase production by *T. aurantiacus*. The findings showed that xylose can be used as an inducerthat leads to a secretion of cellulases and xylanases in 2 litre and 19 litre bioreactors with a fed-batch cultivation strategy[9]. Together with gene editing technologies the production of cellulase degrading enzymes can be further improved.

5. Outlook

The established CRISPR/Cas9 system can now be used to target any known genes in *T. aurantiacus* that are involved in cellulase production to uncover the effect of gene regulation. The corresponding strain cellulase production of gene deletions can then be analyzed with enzyme and growth assays and this knowledge can be used to create strains with multiple mutations to find synergistic effects for further increasing enzyme production. More data about the cellulase and xylanase regulation of *T. aurantiacus* is vital to uncover how this fungus is similar or different to other known fungal species. An interesting target gene is the carbon catabolite repressor gene *creA.* This gene was found in several fungal species that represses polysaccharide degrading genes. The deletion of this gene in *T. aurantiacus* with the help of genetic engineering methods is expected to have a positive effect on the cellulase production[34]. Furthermore, the developed crossing protocol can be used to cross mutant *T. aurantiacus* strains to stack gene mutations and create strains with multiple mutations within a short period of time to find synergistic effects to further increasing enzyme production.

Also, other selection markers can be established using the CRISPR/Cas9 technology. A promising approach is to target and delete the antibiotic resistance gene *amdS* and select for mutant strains on fluoroacetamide plates as is was already successfully performed with the thermophilic fungus *M. thermophila* using CRISPR/Cas9[28]. *T. aurantiacus* WT strains are

expected not to grow on FAA whereas transformants with a deficient *amdS* gene are expected to gain resistance and can therefore be easily selected on FAA plates.
All this knowledge will enable the design of improved strain and bioprocess engineering strategies for cost-efficient enzyme production to help convert plant biomass into biofuels and bio-products.

6. References

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Appendix

Supplement Table 1: Primer list

Primer name	Sequence 5'-3'	Use
LLK687 forward	TAATACGACTCACTATAGCT TTTGCGCGCGAGCGCCGT	gRNA DNA template assembly (sgRNA1)
LLK688 reverse	TTCTAGCTCTAAAACACGGC GCTCGCGCGCAAAAG	gRNA DNA template assembly (sgRNA1)
LLK689 forward	TAATACGACTCACTATAGGA GTCTTCCTGCACAGGCCT	gRNA DNA template assembly (sgRNA2)
LLK690 reverse	TTCTAGCTCTAAAACAGGCC TGTGCAGGAAGACTC	gRNA DNA template assembly (sgRNA2)
LLK691 forward	TAATACGACTCACTATAGTC GGCGCCCGACTTCCCCTA	gRNA DNA template assembly (sgRNA3)
LLK692 reverse	TTCTAGCTCTAAAACTAGGG GAAGTCGGGCGCCGA	gRNA DNA template assembly (sgRNA3)
LLK683 forward	TTCTTACTACAACTTGGCAA CCTTC	*pyrG* amplification
LLK686 reverse	ACAAGCCAAATTACCAGCA GAATAC	*pyrG* amplification
LLK693 forward	GGGTTTAAUGCGTAAGCTCC CTAATTGGC	Fragment 1 for USER cloning
LLK694 reverse	AGCTTACUCGTTTCGTCCTC ACGGACTCATCAGCTTTTGC GGTGATGTCTGCTCAAGCGG	Fragment 1 for USER cloning sgRNA1
LLK695 forward	AGTAAGCUCGTCCTTTTGCG CGCGAGCGCCGTGTTTTAGA GCTAGAAATAGC	Fragment 2 for USER cloning sgRNA1
LLK696 reverse	GGTCTTAAUGAGCCAAGAG CGGATTCCTCA	Fragment 2 for USER cloning
LLK697 reverse	AGCTTACUCGTTTCGTCCTC ACGGACTCATCAGTCGGCGC GGTGATGTCTGCTCAAGCGG	Fragment 1 for USER cloning sgRNA3
LLK698 forward	AGTAAGCUCGTCTCGGCGCC CGACTTCCCCTAGTTTTAGA GCTAGAAATAGC	Fragment 2 for USER cloning sgRNA3
pllk699	GCGTAAGCTCCCTAATTGGC	sgRNA amplification
Pllk700	GGTCTTAATGAGCCAAGAGC G	sgRNA amplification
pllk701	ATGCCTGAACTCACCGCGAC	*hph* gene amplification
pllk702	CTATTCCTTTGCCCTCGGAC G	*hph* gene amplification
PJ1	ggaatccgctCGTAAGCTCCCTAA TTGG	sgRNA Fragment PCR for Gibson assembly
PJ2	ctccgcatgaTATTGGGATGAATT TTGTATGC	sgRNA Fragment PCR for Gibson assembly
PJ3	catcccaataTCATGCGGAGCGGT CAGG	pTS57 Fragment PCR for Gibson assembly
PJ4	ggagcttacgAGCGGATTCCTCA GTCTCG	pTS57 Fragment PCR for Gibson assembly

TS222	CGTAGTACCTGAGCACCCCT CTGAGCTCTT	*hph* verification
TS223	CCATTTGTCTCAACTCCGGA GCTGACATCGA	*hph* verification
pllk699 forward	GCGTAAGCTCCCTAATTGGC	sgRNA verification
pllk700 reverse	GGTCTTAATGAGCCAAGAGC G	sgRNA verification
LLK683 forward	TTCTTACTACAACTTGGCAA CCTTC	*pyrG* verification
LLK686 reverse	ACAAGCCAAATTACCAGCA GAATAC	*pyrG* verification
RG75 forward	GACGGTTTCTATACAGTCTT TTCAG	*pyrE* verification
RG76 reverse	CCCCCGATGTTACTCCGC	*pyrE* verification

Vogel's 50X Salts:

From Microbial Genetics Bulletin 13:42-43, 1956

Na3 Citrate 5.5 H20	150 g
KH2PO4 anhydrous	250 g
NH4NO3 anhydrous	100 g
MgSO4 7 H20	10 g
CaCl2 2 H20	5 g
Trace Element Solution (see below)	5 ml
Biotin Solution (see below)	2.5 ml
Distilled water to make a final volume of 1 L	

2 ml Chloroform was added as a preservative. Store at room temperature.

Trace Element Solution	In 95 mL distilled water, dissolve successively with stirring at room temperature:
Citric acid, 1 H20	5. 00 g
ZnSO4, 7 H20	5. 00 g
Fe(NH4)2(SO4)2, 6 H20	1. 00 g
CuSO4, 5 H20	0. 25 g
MnSO4, 1 H20	0. 05 g
H3BO3, anhydrous	0. 05 g
Na2MoO4, 2 H20	0. 05 g

Add 2 mL Chloroform as a preservative. Store at room temperature.

Biotin solution:
The biotin solution is prepared by dissolving 5.0 mg biotin in 50 mL distilled water. The solution obtained is dispensed in test tubes and stored at -20 °C.

Induction media:
Liquid and solid IM was prepared as followed:

for liquid IM	**For 1L**
Reagent	**Volume needed [mL]**
20X AB* salts solution	50
500 mM phosphate solution	2.4
MES-Buffer 50X	20
10 % thiamine	0.1
36 % glucose	5.5
acetosyringone (100 mM)	*3*
water	919

for solid IM	**For 1L**
Reagent	**Volume needed [mL]**
20X AB* salts solution	50
500 mM phosphate solution	2.4
MES-Buffer 50X	20
10 % thiamine	0.1
36 % glucose	5.5
acetosyringone (100 mM)	*3*
water	479
Agar in water	15 g in 440 mL

Supplementary Table Chemicals, Enzymes, Kits and Laboratory Equipment:

Chemicals	**Supplier**	**Product ID**
Potato Dextrose Agar Plates. 100 mm Plates, 20 Plates/Sleeve, Sterile.	TEKNOVA	P0047
Hygromycin B	VWR Life Science	J607-100MG
5-Fluorootic Acid	Research Products International	F10501-5.0
Tween80	Sigma	P5188-100ML
Cefotaxime Sodium Salt	TCI Tokyo Chemical Industry	C2224
Certified Nuclease Free Water	Growcells.com	17DC1020D
3',5'-Dimethoxy-4'-hydroxy-acetophenone	Sigma-Aldrich	D134406-1G
Thiamine hydrochloride	SIGMA	T1270-25G
HEPES sodium salt	Sigma	H3784-100G
GelRed	Biotum	41003-1
50X TAE Buffer	BioRad	1610773
Phusion U Hot Start DNA Polymerase	thermo fisher scientific	F-555S
Dimethyl sulfoxide, 99 %	BTC Beantown Chemical	132780-500G
Glycerol	VWR	BDH1172-1LP
Chloroform	Sigma-Aldrich	37978-100ML
Difco Potato Dextrose Agar	BD Becton, Dickinson and Company	213400
Bacto Agar	BD	214010
Agarose	Fisher BioReagents	BP160-500
Luria-Bertani medium		
Ultra pure Uracil	VWR Life Sciences	0847-250G
Soy Peptone, GMO-free, animal-free	Amresco	N454-500G
Difco Saccharose	BD Becton, Dickinson and Company	217610
Potato Dextrose Broth	Fluka Analytical	P6685-250G
Difco Oatmeal Agar	BD Becton, Dickinson and Company	7297517
LB Agar plates with Carbenicilin-100	TEKNOVA	L1010
LB Agar Plates with Kanamycin-50	TEKNOVA	L1025
Enzymes	**Supplier**	**Product ID**
PacI	NEB	R0547S
Nt.BbvCI	NEB	R0632S
USER® Enzyme	NEB	M5508

High Efficiency Transformation Protocol using NEB® 10-beta Competent E. coli (High Efficiency) (C3019)	NEB	C3019
SIG10 5-alpha Chemically Competent Cells	Sigma-Aldrich	
Phusion® High-Fidelity DNA Polymerase	NEB	M0530L
Q5 Hot Start High-Fidelity DNA Polymerase	NEB	M0493S
LongAmp Taq 2X Master Mix	NEB	M0287
FastDigest DpnI	Thermo Fisher Scientific	FD1703
Kit Name	**Function**	**Supplier**
QIAprep Spin Miniprep Kit (250)	Plasmid purification	Qiagen
QIAquick PCR Purification Kit	PCR purification	Qiagen
Invitrogen GeneArt™ Precision gRNA Synthesis Kit	gRNA synthesis	ThermoFisher Scientific
Guide-it™ sgRNA In Vitro Transcription and Screening Systems User Manual	Cas9 cleavage Assay	Takara Bio USA, Inc.
QIAEX II Gel Extraction Kit	gDNA extraction from agarose gels	Qiagen
Maxwell® RSC Instrument (AS4500)	DNA Extraction	Promega
Gibson Assembly Cloning Kit	Gibson Assembly	NEB
NEB Gibson Assembly Cloning Kit	NEB	5510S
Device	**Supplier**	**Application**
NanoDrop™ 2000/2000c Spectrophotometers	Thermo Scientific™, USA	Quantification and assessment of purity of DNA or RNA
Allegra X-15R Centrifuge	Beckman Coulter, USA	pelleting cell cultures
Vista Vision microscope	VWR Life Science	Spore counting
Bright-Line™ Hemacytometer	Sigma-Aldrich	Spore counting
VWR 250V Electrophoresis Power Supply	VWR Life Science	DNA electrophoresis
Eppendorf™ 5424 Microcentrifuge	eppendorf	Cell pelleting
Veriti 96 Well Thermal Cycler	Applied Biosystems	PCR, Incubation
Thermomixer	eppendorf	Incubation
Vortex Mixer	VWR	Homogenization
FastPrep-24™ 5G Instrument	MP Biomedicals	Bead beating, Lysis of fungi
Maxwell® RSC Instrument (AS4500)	Maxwell	gDNA extraction
Gene Pulser Xcell™ Electroporation System	BioRad	Electroporation

2mm VWR electroporation cuvette	VWR	Electroporation
Miracloth	Millipore Sigma	475855-1R
Zirconia/Silica Beads 0.5mm	Biospec Products	11079105z
Yttria Stabilized Zirconia (YSZ) Grinding Media Sperical 5.0 mm	Inframat Advanced Materials	4039GM-S050
FastPrep-24™ 5G Instrument	MP BioMedicals	Bead beating

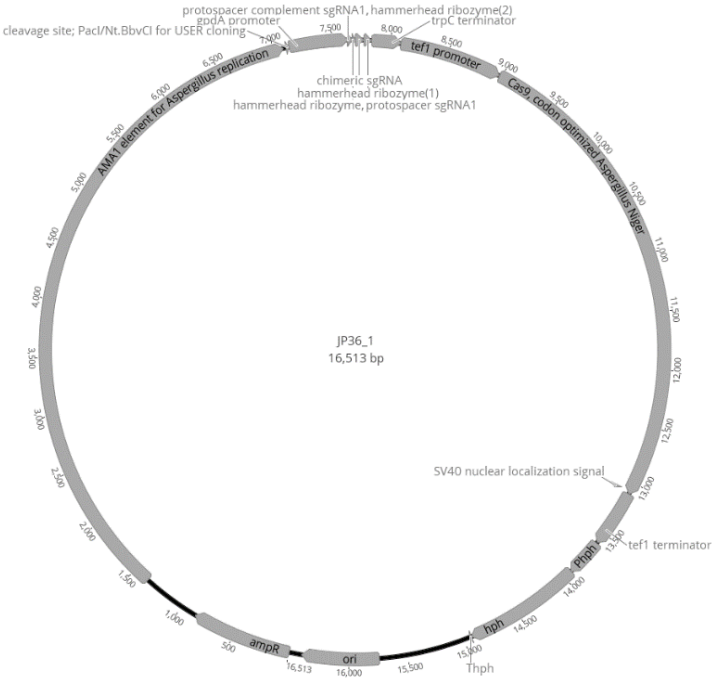
Protocol for colony PCR using LongAmp Taq 2X Master Mix:

Component	**50 µL reaction**	**Final Conc.**
10 µM Forward Primer	2 µL	0.4 µM (0.05–1 µM)
10 µM Reverse Primer	2 µL	0.4 µM (0.05–1 µM)
Template DNA	2 uL	<1,000 ng
LongAmp *Taq* 2X Master Mix	25 µL	1X
Nuclease-free water	34 µL	

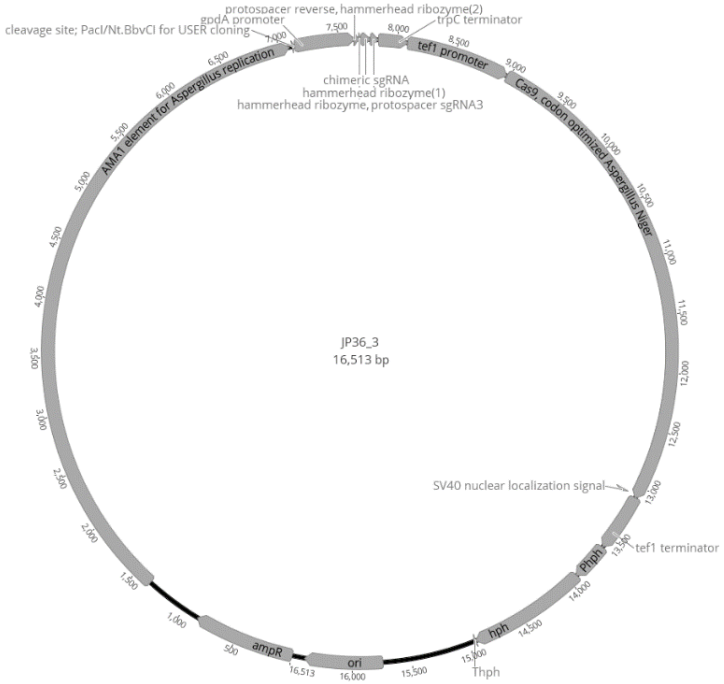
Thermocycling conditions for a LongAmp Taq 2X Master Mix:

STEP	**TEMP**	**TIME**
Initial Denaturation	94 °C	30 seconds
30 Cycles	94 °C 45-65 °C 65 °C	30 seconds 60 seconds 50 seconds per kb
Final Extension	65 °C	10 minutes
Hold	4-10 °C	

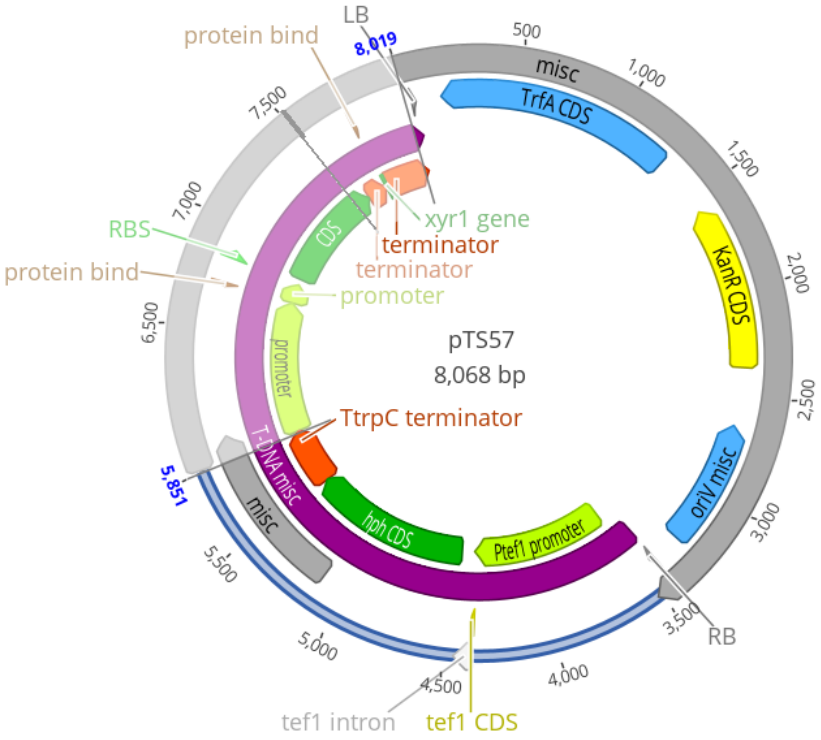
Plasmid maps obtained from Geneious software 11.1.2:



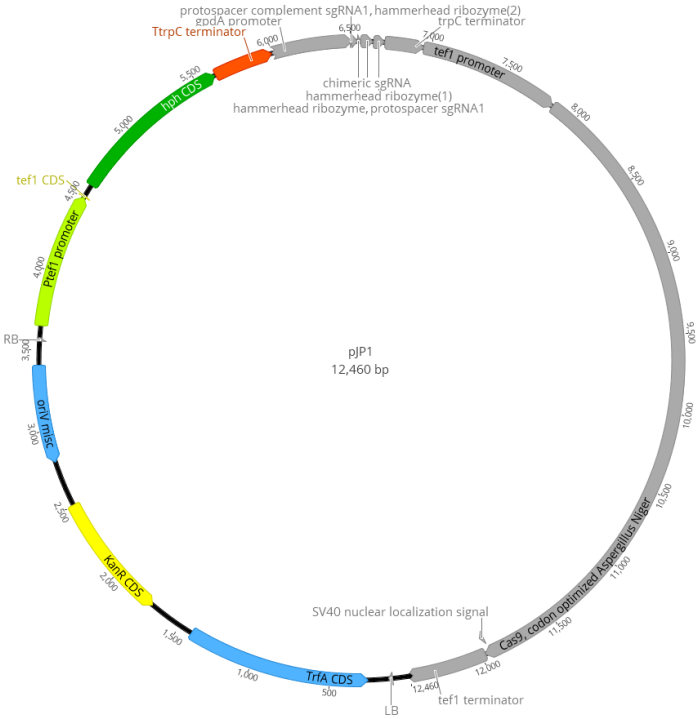
Supplement Figure 1: Plasmid JP36_1 harboring sgRNA1 used for Gibson Assembly with plasmid pTS57



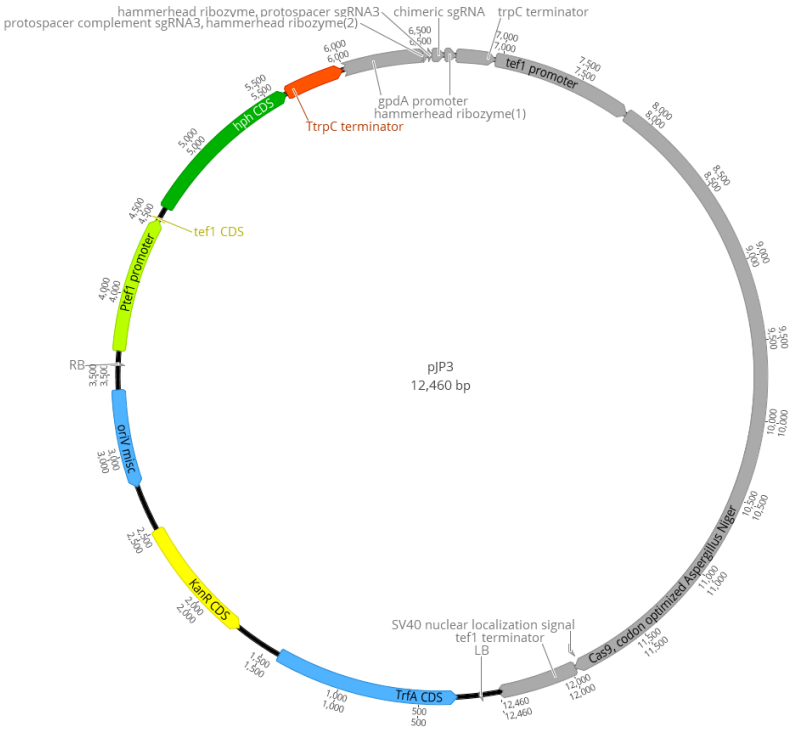
Supplement Figure 2: Plasmid JP36_3 harboring sgRNA3 used for Gibson Assembly with plasmid pTS57



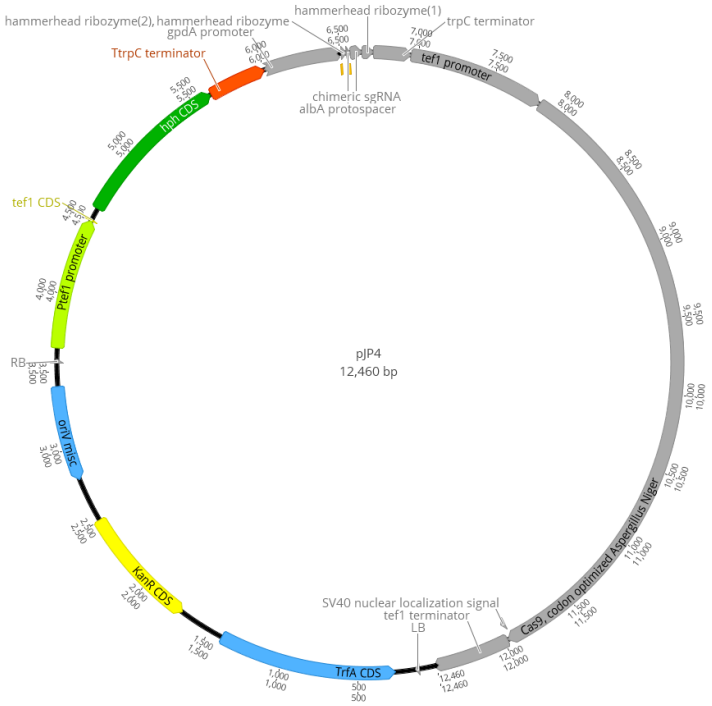
Supplement Figure 3: Plasmid pTS57 used for Gibson Assembly with plasmid JP36_1 and JP36_3



Supplement Figure 4: CRISPR/Cas9 plasmid pJP1 harboring sgRNA1 targeting pyrG in T. aurantiacus used for ATMT



Supplement Figure 5: CRISPR/Cas9 plasmid pJP3 harboring sgRNA3 targeting pyrG in T. aurantiacus used for ATMT



Supplement Figure 6: CRISPR/Cas9 plasmid pJP4 harboring sgRNA targeting albA in A. niger used for ATMT