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Establishment of CRISPR/Cas9 in *Alternaria alternata*

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ABSTRACT

The filamentous fungus *Alternaria alternata* is a potent producer of many secondary metabolites, some of which like alternariol or alternariol-methyl ether are toxic and/or cancerogenic. Many Alternaria species do not only cause post-harvest losses of food and feed, but are aggressive plant pathogens. Despite the great economic importance and the large number of research groups working with the fungus, the molecular toolbox is rather underdeveloped. Gene deletions often result in heterokaryotic strains and therefore, gene-function analyses are rather tedious. In addition, *A. alternata* lacks a sexual cycle and classical genetic approaches cannot be combined with molecular biological methods. Here, we show that CRISPR/Cas9 can be efficiently used for gene inactivation. Two genes of the melanin biosynthesis pathway, *pksA* and *brm2*, were chosen as targets. Several white mutants were obtained after several rounds of strain purification through protoplast regeneration or spore inoculation. Mutation of the genes was due to deletions from 1 bp to 1.5 kbp. The CRISPR/Cas9 system was also used to inactivate the orotidine-5-phosphate decarboxylase gene *pyrG* to create a uracil-auxotrophic strain. The strain was counter-selected with fluor-orotic acid and could be re-transformed with *pyrG* from *Aspergillus fumigatus* and *pyr-4* from *Neurospora crassa*. In order to test the functioning of GFP, the fluorescent protein was fused to a nuclear localization signal derived from the StuA transcription factor of *Aspergillus nidulans*. After transformation bright nuclei were visible.

1. Introduction

Alternaria alternata is an economically very important fungus, because of its potential to cause tremendous post-harvest losses due to toxin contamination of food and feed (Kabak et al., 2006; Lee et al., 2015; Moretti et al., 2017). The species belongs to the so-called black molds and is a potent producer of alternariol and its derivatives but also many other secondary metabolites (Bräse et al., 2009; Rodriguez-Carrasco et al., 2016). Alternariol is a cancerogenic compound and thus the content in food and feed should be monitored (Fleck et al., 2016). The understanding of its toxicity may be further complicated through combinatory effects of different compounds (Vejdovszky et al., 2016). In addition to their potential to contaminate food and feed with mycotoxins, many strains are infecting living plants (Cho, 2015; Logrieco et al., 2009; Tsuge et al., 2013).

Despite the importance of the genus *Alternaria*, the spectrum of molecular biological methods is not yet fully developed in all species. For instance, in our hands it proved very difficult to create clean gene-deletion strains in *A. alternata*, although several examples show that homologous recombination and thus gene-replacements work (Estiarte et al., 2016; Pruß et al., 2014; Yamagishi et al., 2006). A frequent problem using traditional gene-replacement methods with one kb flanking regions is the formation of heterokaryons and since *A. alternata* lacks a sexual cycle and produces multi-cellular asexual spores, isolation of clonal progeny is difficult. Therefore, we aimed at establishing the CRISPR/Cas9 system. In this system the Cas9 endonuclease is guided to a chosen DNA sequence with the help of a guide RNA. Both, the Cas9 enzyme and the guide RNA are introduced into fungi by transformation. The CRISPR (clustered regularly interspaced short palindromic repeats) system has been adapted for gene editing in many organisms and revolutionized many fields (Cho et al., 2013; Jinek et al., 2013). It was established first in the model fungus *Aspergillus nidulans* and in several other Aspergillus species (Jakociunas et al., 2016; Krappmann, 2017; Nodvig et al., 2015). In similar approaches the functioning of the system was shown in *A. fumigatus*, where in addition to gene inactivation, demonstrated by the inactivation of melanin biosynthesis genes, the insertion of GFP was achieved by microhomology-mediated end joining (MME) (Fuller et al., 2015). The beauty of the latter system is, that genes can be tagged without an additional marker gene

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(Zhang et al., 2016). In several other fungi CRISPR/Cas9 has been applied and although the principle mechanisms and procedures are similar for each experimental system, adaptations are always necessary (Liu et al., 2017; Schuster et al., 2016). After proof-of principle publications, the system has been used for functional analyses, such as the analysis of secondary metabolite gene clusters or the engineering of enzyme-producing strains (Kuivanen et al., 2016; Liu et al., 2017; Nielsen et al., 2017; Weber et al., 2017).

In this study, we used the CRISPR/Cas9 system established in *A. nidulans* and adapted it for the use in *A. alternata*. We have used the tool to inactivate two genes of the melanin-biosynthesis pathway and in addition created a *pyrG* auxotrophic mutant. The latter should solve the problem, that often gene-inactivation is not followed by re-complementation of the defect with a wild-type copy of the gene, due to the lack of different selection markers. Furthermore, we show that fluorescent proteins can be used in *A. alternata*.

2. Materials & methods

2.1. Protoplast transformation of A. alternata

Fungal spores were harvested from a mCDB culture plate and inoculated into 100 ml liquid mCDB (4% glucose, 0.1% yeast extract, 0.1% $NaNO_3$, 0.025% NH_4Cl, 0.1% KH_2PO_4, 0.025% KCl, 0.025% NaCl, 0.05% $MgSO_4 \cdot (7H_2O)$, 0.001% $FeSO_4$, 0.001% $ZnSO_4$, 1.5% agar) for overnight cultivation at 28 °C and 180 rpm. The mycelium was harvested by filtering, washed with 0.7 M NaCl and digested in a Kitalase (Wako Chemicals) suspension (150 mg in 15 ml 0.7 M NaCl) for 1 h with soft shaking at 120 rpm at 30 °C. Protoplast quality and quantity were checked by microscopy. Protoplasts were separated from cell fragments by filtering through two layers of Miracloth and precipitated at 560*g* for 10 min at room temperature. The Kitalase solution was discarded and the protoplasts were washed once with ice cold 0.7 M NaCl and resuspended in 100 µl STC (1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 8). 5 µg of plasmid DNA were added to the protoplasts followed by a 10 min incubation on ice. DNA uptake was induced with a heat shock at 42 °C for 5 min and, after a 5 min incubation step on ice, 800 µl 40% PEG (40% polyethylene glycol [PEG] 4000, 50 mM Tris-HCl [pH 8], 50 mM $CaCl_2$) was added to the protoplasts, followed by 15 min incubation at room temperature. The suspension was mixed with 50 ml warm regeneration medium (34% sucrose, 0.5% yeast extract, 0.5% casein hydrolysate, 0.75% agar) and split into two petri dishes. After over-night incubation at 28 °C the transformation plates were overlayed with 15 ml warm regeneration medium containing hygromycin (80 µg/ml).

2.2. Plasmid construction

The CRISPR/Cas9 vectors with specific sgRNA genes, containing the respective protospacer sequences as well as a 6 bp inverted repeat of the 5′-end of the protospacer to complete the hammerhead cleavage site, were generated in a single cloning step. New protospacer sequences were inserted into the linearized pFC332 vector by combining two PCR fragments amplified from plasmid pFC334 and the pFC332 vector in a NEBuilder reaction. The primers, which contain the variable regions, used to generate the two sgRNA gene fragments, were obtained from MWG-Eurofins and are listed in Table 1. The amplified fragments were flanked by 30 bp complementary sequences to each other and the linearized vector in order to generate the functional vectors in a single NEBuilder reaction (New England Biolabs, Frankfurt). The fragments were amplified from pFC334 with proofreading polymerase Q5 (NEB) by a touch-down PCR program [Denaturation: Initial step for 3 min at 98 °C, all following steps for 20 s at 98 °C; Annealing: 5 cycles at 67 °C for 20 s, 5 cycles at 65 °C for 20 s, 25 cycles at 63 °C for 20 s; Elongation: 10 s at 72 °C]. Standard reaction volumes were 50 µl including 1× Q5 reaction buffer, 200 µM dNTPs, 0.5 µM primers, 1 U Q5 and 100 ng of plasmid DNA. Plasmid pFC332 was linearized using *Pac*I and assembled with the PCR fragments, following the NEBuilder protocol. *E. coli* transformation and plasmid isolation was done according to standard protocols (Sambrook and Russel, 1999).

3. Results and discussion

Molecular biological tools are well-developed for many model fungal systems. However, for many economically important fungi, such as *A. alternata*, methods are underdeveloped and especially gene-knock-out procedures are tedious or impossible. Here, we show that CRISPR/Cas9 can be efficiently used for gene inactivation in *A. alternata*. In addition, we established *pyr4* as a selection marker and GFP for protein tagging.

Table 1
List of oligonucleotides used in this study. The red letters indicate the protospacer sequences.

Oligonucleotide	Sequence 5′ → 3′
Crispy 2.0 fwd	GGTCATAGCTGTTTCCGCTGA
Crispy 2.0 rev	TGATTCTGCTGTCTCGGCTG
Proto pksA fwd	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCCGACCAGTACCCGCTCCTGGTTTTAGAGCTAGAAATAGCAAGTTAAA
Hh pksA rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCCGACCCGGTGATGTCTGCTCAAGCG
Proto brm2 fwd	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGACATGGAGCCTTGCCGGCAGTTTTAGAGCTAGAAATAGCAAGTTAAA
Hh brm2 rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGACATGCGGTGATGTCTGCTCAAGCG
Proto pyrG fwd	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGTCAGCCGAACTCCTACAACGTTTTAGAGCTAGAAATAGCAAGTTAAA
Hh pyrG rev	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGTCAGCCGGTGATGTCTGCTCAAGCG
PksA 0.5 kb seq fwd	CATACTGGATCAATCTTTCTTTCCC
PksA 0.5 kb seq rev	CGCAGTTGGCGCATTTCATC
PksA 3 kb fwd	GACGCCGGGTTATGAGCAAG
PksA 3 kb rev	CTACTTCATCACAGGTGGTGTG
Brm2 test fw	GCCGATTTCTAGGACACTCC
Brm2 seq fw	GATCTCATGAAACGGACAGG
Brm2 test re	CCATCCACTCTTACTGTGACC
PyrG seq fwd	GACCGTCGACACTAGCTTTC
PyrG seq rev	CGTTGTGAGCGTGACAACTTC