Establishment of CRISPR/Cas9 in *Alternaria alternata*

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**A R T I C L E   I N F O**

Article history:
Received 2 February 2017
Revised 5 March 2017
Accepted 6 March 2017
Available online 10 March 2017

Keywords:
Alternaria
Knock-out
CRISPR
Uracil

**A B S T R A C T**

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.

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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 combinatorial effects of different compounds (Vejdovszyk 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-replacement methods 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 interspersed 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; Lodvig 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...
(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_4·7H_2O, 0.001% FeSO_4·7H_2O, 0.001% ZnSO_4·7H_2O, 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. 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 PacI 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

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<tr>
<td>Crtpy 2.0 rev</td>
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3.1. Establishment of CRISPR/Cas9 for gene inactivation

The CRISPR/Cas technology has only been adapted to several fungi. In order to make use of this powerful technique in A. alternata we adapted the system from A. nidulans (Nodvig et al., 2015). As a first proof-of-principle of the functionality of the system, we have chosen the melanin biosynthetic pathway for inactivation and inactivated the polyketide-synthase gene, pksA and the 1,3,8-THN reductase encoding gene, brm2. Inactivation or down regulation of these genes usually results in melanin deficient colonies that appear white or brown, respectively and are easy to identify (Fetzner et al., 2014; Kimura and Tsuge, 1993; Shiotani and Tsuge, 1995). Two plasmids (pCP1 and pMW29) were generated, which contain the Cas9 coding sequence from S. pyogenes (codon optimized for A. niger) and the single-guide RNA (sgRNA) targeting pksA and brm2, respectively. Cas9 is 3’-extended by a sequence encoding a SV40 nuclear localization signal (PKKKRKV). The construct is controlled by the strong constitutive A. nidulans tef1 promoter and tef1 terminator (Kitamoto et al., 1998). The sgRNA sequence is flanked by two ribozyme sequences: hammerhead on the 5’-end and hepatitis delta virus at the 3’-end. The ribozyme sequences ensure the liberation of the sgRNA from the transcript in the nucleus. The expression of the sgRNA-containing transcript is controlled by the strong constitutive A. nidulans gpdA promoter and the trpC terminator. The plasmid is a self-replicating plasmid and does not need to be integrated into the genome. This allows for easy isolation of cured strains, after growth on non-selective medium. The resulting strains are again hygromycin sensitive and can be transformed again using the same selection marker.

A 20-nucleotide long protospacer sequence with the 3’-PAM CGG for pksA and AGG for brm2 respectively was chosen at the start of the ORF (nucleotide +32 to +51 pksA and +18 to +37 brm2). The protospacer sequences were introduced into plasmid pFC332 by PCR and homologous recombination (see Materials and Methods). The resulting plasmids pCP1 and pMW29 were used to transform A. alternata ATCC 66981 via protoplast transformation. The resulting transformants were transferred to selective medium containing hygromycin B (80 μg/ml). On average one out of ten transformants showed the expected phenotype lacking melanin production with white colonies when pksA was targeted (Fig. 1) or brown colonies for brm2 mutants (Fig. 2). However, some colonies that showed wild-type melanin production on the primary transformation plate developed melanin deficient colonies after a few days of growth (Fig. 1), indicating successive inactivation of the genes. On the other hand for some white colonies we observed that wild-type colonies appeared again, when transferred to non-selective medium. This indicated that the original transformants were heterokaryotic with transformed and non-transformed nuclei. In order to generate stable melanin-mutant strains, colonies were grown from single spores or from protoplasts. In the case of the pksA knock-out, the primary mutant was re-grown two times on hygromycin-containing medium and two times on non-selective medium. Growth on hygromycin-free medium led to the loss of

![Fig. 1. Inactivation of the melanin pathway by targeting of pksA using CRISPR/Cas9.](image-url)

(A) Transformation of the plasmid pCP1 containing cas9 and a sgRNA targeting pksA results in melanin deficient colonies that produce non-melanized conidia and hyphae. The arrows indicate pksA mutants. A colony with wild type melanin production is encircled. (B) Due to the constitutive activity of cas9 and the sgRNA, colonies with wild-type appearance can be grown for longer times on selective medium to develop the mutation over time. The plate was inoculated with spores from a green colony. A mixture of green and white colonies appeared. (C) Sequence analysis of one pksA-mutant strain revealed a 1589 bp deletion with 724 bp of the ORF and 865 bp of the 5’-UTR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the transformation plasmid and thereby loss of Cas9 and the selection marker. For other genes, not described in this paper, three rounds of re-streaking were necessary to obtain a stable and plasmid-free strain. The confirmation of a monokaryotic strain of *A. alternata* is especially important, if genes are analyzed whose inactivation does not lead to obvious phenotypic changes.

Since the introduced double strand break through Cas9 leads to random mutations, due to the error prone repair mechanism of non-homologous end joining, the white mutants were further examined. Genomic DNA was extracted and used as template to amplify a 1 kb fragment with primers binding 500 bp up- and downstream of the respective protospacer sequence. For some mutants the resulting bands showed the expected size of one kb while for others smaller bands were obtained. Sequence analysis of the corresponding PCR fragments revealed deletions of one to 303 base pairs (Fig. 2). For some mutants we were not able to amplify the genomic region, suggesting larger deletion events. Indeed, amplification of a 4 kb genomic region resulted in a 2.5 kb PCR fragment compared to the control. Sequence analysis confirmed the 1.5 kb deletion (Fig. 1).

In comparison to traditional deletion approaches, the CRISPR/Cas9 mutagenesis approach has the big advantage, that the system

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**Fig. 2.** Deletion events in *brm2* mutants obtained with the CRISPR/Cas9 system. (A) To explore the range of deletion events that occur during the use of CRISPR/Cas9, the *brm2* gene involved in melanin production was targeted. In contrast to the white colonies of *pksA* mutants, the *brm2*-mutant strains appear brown. (B) Sequence analysis of the transformants showed deletions of one to 303 base pairs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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**Fig. 3.** Inactivation of the orotidine 5′-phosphate decarboxylase gene *pyrG*. Mutation of *pyrG* using CRISPR/Cas9 leads to uracil-auxotrophic mutants, which are unable to grow on medium without uracil supplementation (Δ*pyrG*). A plasmid containing *pyr4* from *N. crassa* was used for re-complementation (rec.) of the auxotrophy.
remains active and successively mutagenizes the target gene. Hence isolation of homokaryotic strains can be achieved by prolonged incubation of the colonies in addition to the isolation of clonal progeny from spores or protoplasts (Fig. 1). Another big advantage of the CRISPR/Cas9 technology is the fact that the self-replicating plasmid will be lost once the selection pressure is removed. What remains in the genome of the mutant is only the modification at the gene locus of interest. Hence, the cured strain may be transformed again using the same selection marker to obtain double-mutant strains. This is especially important in assexual species like A. alternata, where strains cannot be constructed through sexual crosses.

3.2. Establishment of a uracil-auxotrophic mutant strain

So far selection of A. alternata transformants was achieved with dominant markers such as hygromycin. In comparison to auxotrophic markers in e.g. A. nidulans, selection with hygromycin is less efficient. Therefore, we used the CRISPR/Cas9 technology to create a uracil-auxotrophic mutant strain by inactivation of the orotidine 5'-phosphate decarboxylase gene, pyrG. Auxotrophic mutants are only able to grow on medium supplemented with uracil and uridine. In addition, negative selection can be performed by using 5-fluorourotic acid (5-FOA) since remaining wild-type copies lead to growth inhibition due to the conversion of 5-FOA to toxic 5-fluouracil by PyrG. A pyrG orthologue was identified in the genome of A. alternata (Dang et al., 2015). The 379 amino acid protein showed a similarity of 44% to PyrG from Aspergillus fumigatus, 44% to A. nidulans and 41% to Pyr4 from Neurospora crassa. Using the same strategy as for the inactivation of the melanin biosynthetic genes, the pyrG gene of A. alternata was inactivated. From 24 primary transformants, six grew on medium supplemented with 5-FOA, although they were still growing slower than without 5-FOA. This could have been an indication for heterokaryosis. However, the strains did not grow on medium without uracil and uridine (Fig. 3).

In order to test the suitability of heterologous pyrG genes for the re-complementation of the A. alternata pyrG mutant, we used the pyrG gene from A. fumigatus and the pyr-4 gene from N. crassa. In both cases transformants were obtained (Fig. 3). As compared to the selection with hygromycin, the background of non-transformed strains was much lower.

3.3. Establishment of GFP

Since the discovery of the green fluorescent protein (GFP), fluorescent proteins became a powerful tool in modern biology (Chalfie et al., 1994; Misteli and Spector, 1997; Suellmann et al., 1997). Until now no microscopic studies of the A. alternata strain ATCC66981 have been done using any of them. After the establishment of auxotrophic markers via the CRISPR/Cas9 technology we were able to introduce a plasmid containing the marker gene pyr-4 and GFP under the strong constitutive promoter gpdA. Inspection of non-transformed A. alternata hyphae revealed strong autofluorescence in some compartments. In order to distinguish specific GFP signals from such autofluorescence, GFP was C-terminally tagged with a nuclear localization signal (NLS) of stuA from A. nidulans (Suelmann et al., 1997). To quantify the transformation efficiency we screened transformants microscopically. After 24 h incubation at 28 °C in 8-well microscopy slide, 9 out of 14 transformants showed green1 fluorescent nuclei, in all hyphal compartment (Fig. 4). Nuclei of the hyphae were counter-stained using Hoechst 33342.

Fig. 4. Nuclear localization of GFP in A. alternata. (A) To test the functionality of GFP in A. alternata ATCC66981, we used a construct containing pyr# from N. crassa as selection marker and GFP under the control of the constitutive A. nidulans gpdA promoter. GFP was C-terminally tagged with the NLS of stuA from A. nidulans. (B) Transformants showed a strong GFP signal in nuclei, which was confirmed by Hoechst 33342 staining of the hyphae (upper panels). Wild-type hyphae only showed autofluorescence (lower panels).

Acknowledgements

We thank Uffe Mortensen (DTU, Lyngby, Denmark) for providing us plasmids pFC332 and pFC334. This work was supported by BMBF NANOKAT II (No. 031A613B). We would like to thank Christopher Lawrence (Virginia Tech, Blacksburg, VA, USA) for access to the A. alternata sequence database.

References


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