Microbiological Research 272 (2023) 127397

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Inheritable CRISPR based epigenetic modification in a fungus

Xiaoyi Chen, Juan P. Moran Torres, Yiling Li, Luis G. Lugones, Han A.B. Wösten *

Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands

ARTICLE INFO

Keywords:
Fungus
Aspergillus
Epigenetic modification
Spore production

ABSTRACT

The CRISPRoff system was recently introduced as a programmable epigenetic memory writer that can be used to silence genes in human cells. The system makes use of a dead Cas9 protein (dCas9) that is fused with the ZNF10 KRAB, Dnmt3A, and Dnmt3L protein domains. The DNA methylation resulting from the CRISPRoff system can be removed by the CRISPRon system that consists of dCas9 fused to the catalytic domain of Tet1. Here, the CRISPRoff and CRISPRon systems were applied for the first time in a fungus. The CRISPRoff system resulted in an inactivation up to 100 % of the target genes *flbA* and *GFP* in *Aspergillus niger*. Phenotypes correlated with the degree of gene silencing in the transformants and were stable when going through a conidiation cycle, even when the CRISPRoff plasmid was removed from the *flbA* silenced strain. Introducing the CRISPRon system in a strain in which the CRISPRoff plasmid was removed fully reactivated *flbA* showing a phenotype similar to that of the wildtype. Together, the CRISPRoff and CRISPRon systems can be used to study gene function in *A. niger*.

1. Introduction

CRISPR/Cas9 is widely used to edit genes. However, the double stranded DNA breaks resulting from Cas9 may lead to genome rearrangements and gene loss (Cullot et al., 2019). Recently, a CRISPRoff system was developed that can be used to silence genes in human cells without introducing DNA breaks (Nuñez et al., 2021). This system is a programmable epigenetic memory writer that establishes DNA methylation and repressive histone modifications. The epigenetic changes resulting from transient expression of the CRISPRoff system are maintained for more than 450 cell divisions, showing this form of gene silencing is stable and inheritable (Nuñez et al., 2021). The CRISPRoff system is composed of a dead Cas9 (dCas9) that is fused with the human ZNF10 KRAB and the Dnmt3A (D3A) and Dnmt3L (D3L) protein domains. D3A methylates DNA, while its binding partner D3L maintains DNA methylation (O'Geen et al., 2019). The transcriptional repressor domain Krüppel-associated box (KRAB) is found in about one third of the zinc-finger proteins in the human genome, while it is absent in fungal genomes (Urrutia, 2003). KRAB interacts with KAP1, which is a scaffold protein recruiting histone modification proteins, leading to heterochromatin (Lupo et al., 2013). The DNA methylation resulting from the CRISPRoff system can be removed by the CRISPRon system. This system is based on TETv4 (Nuñez et al., 2021), which consists of an 80 amino acid XTEN80 linker flanked by dCas9 and the catalytic domain of TET1 (Nuñez et al., 2021). TET1 is part of the TET (ten-eleven translocation) family enzymes, which have been used for programmable demethylation of promoters of human genes, thus resulting in gene activation (Holtzman and Gersbach, 2018; Maeder et al., 2013; Xu et al., 2016).

In this study, genes *flbA* (under control of its own promoter) (Wieser et al., 1994; Yu et al., 1996; Krijgsheld et al., 2013) and *GFP* (under control of the *glaA* promoter) (Siedenberg et al., 1999) were used to assess the CRISPRoff and CRISPRon systems in *A. niger*. Both genes have an easily screenable phenotype. Decreased *GFP* and *flbA* expression results in reduced fluorescence and decreased sporulation, respectively. FlbA is a RGS domain protein that stimulates GTPase activity of the Gα-subunit FadA. As a consequence, it promotes formation of the Gα-Gβ-Gγ trimer thereby repressing hyphal proliferation and stimulating asexual development. Genes *flbA* and *GFP* were silenced in *A. niger* with an efficiency up to 100 %. Expression of the *flbA* gene could be restored by the CRISPRon system. Together, CRISPRoff and CRISPRon can be used to study gene function in *A. niger*.

2. Material and methods

2.1. Strains and culture conditions

Escherichia coli TOP10 was used for constructing plasmids. *A. niger* strains (Table 1) were routinely grown at 30 °C on minimal glucose medium (70.6 mM $NaNO_3$, 11 mM KH_2PO_4, 6.7 mM KCl, 2 mM

* Corresponding author.
E-mail address: h.a.b.wosten@uu.nl (H.A.B. Wösten).

https://doi.org/10.1016/j.micres.2023.127397
Received 3 March 2023; Received in revised form 26 April 2023; Accepted 26 April 2023
Available online 29 April 2023

Table 1
A. niger strains used in this work.

Strain	Genotype	Reference
N400		CBS 120.49
Δ*flbA*	*uncA*$^-$, *pyrG*$^+$, Δ*flbA*::*hph*	Krijgsheld et al. (2013)
N400pyrG378	*pyrG378*$^-$	Unpublished data
AR9#2	*uncA*$^-$, *pyrG*$^+$, P*glaA*:: sGFP(S65T)	Siedenberg et al. (1999)
AR9#2XC	*uncA*$^-$, *pyrG378*$^-$, P*glaA*:: sGFP (S65T)	This study
CFFA1	*uncA*$^-$, *pyrG*$^+$	This study
CFFA2	*uncA*$^-$, *pyrG*$^+$	This study
CFGA1	*uncA*$^-$, *pyrG*$^+$	This study
TEFA1	*uncA*$^-$, *pyrG*$^+$	This study
CF(-sgRNA, -KRAB)	*uncA*$^-$, *pyrG*$^+$	This study
CF(-KRAB)	*uncA*$^-$, *pyrG*$^+$	This study
CF(+KRAB)	*uncA*$^-$, *pyrG*$^+$	This study
sgRNA_1–10	*uncA*-, *pyrG*+	This study

$MgSO_4$0.7 H_2O, trace metal solution (Vishniac and Santer, 1957), 1 % glucose), with (MM-GA) or without (MM-G) 1.5 % agar. Spores were collected from 3-day-old confluent potato dextrose agar (PDA) cultures that had been inoculated with a confluent layer of 50,000 conidia. For preparing protoplasts, mycelium was harvested from 16-h liquid shaken cultures (200 rpm) using transformation medium (TM; MM-G with 0.5 % yeast extract and 0.2 % casamino acids). TM was also used to isolate DNA. MM-SA (MM with 327 g L^{-1} sucrose and 1.2 % agar) was used to regenerate protoplasts and to select transformants, while purification of transformants was done on MM-G. To this end, 2 g L^{-1} uridine and 100 μg mL^{-1} hygromycin were added, if necessary. RNA was extracted from mycelium of 3-day-old colonies that had been grown between two polycarbonate membranes (pores of 0.1 μm, diameter 76 mm; Profiltra, Almere, The Netherlands) on MM-GA medium for RT-qPCR assay.

2.2. Construction of plasmids

\The chimaeric gene consisting of KRAB, dCas9 and Dnmt3A-3L (Fig. 1) was obtained from the CRISPRoff V2.1 plasmid (Addgene #167981; Nuñez et al., 2021) using *BglII* and *SmaI*. The sequence was introduced into *StuI* linearized vector pyrG plug 3.0 (Supplemental Table 1) using NEBuilder® HiFi DNA Assembly (NEB), resulting in plasmid pG3.0-CRISPRoff-v2.1 (Fig. 1). The sgRNA sequences targeting the *flbA* (ASPGD ID An02g03160) and *glaA* (ASPGD ID An03g06550) promoters (the latter to control expression of *GFP*) were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) (Supplemental Table 2). Two sgRNAs were designed for *flbA* and one for *glaA* targeting the promoters 802 (*flbA*), 133 (*flbA*), and 129 (*glaA*) bp upstream of the start codon of *flbA* and *GFP*. The *flbA* and *glaA* sgRNA sequences were introduced between the proline tRNA promoter ($tRNA^{pro}$) and terminator ($tRNA^{ter}$) by Gibson assembly. To this end, the promoter was amplified by PCR using primer sgRNA universal-F combined with dcas9-flbA-sgRNA1-P-R, dcas9-flbA-sgRNA2-P-R, or dcas9-glaA-sg RNA1-P-R (Supplemental Table 3). Similarly, the terminator was amplified using primer sgRNA universal-R combined with dcas9-flbA-sgRNA1-T-F, dcas9-flbA-sgRNA2-T-F or dcas9-glaA-sg RNA1-T-F (Supplemental Table 3). The Gibson assembled sgRNA gene was inserted into *PacI* linearized pG3.0-CRISPRoff-v2.1 plasmid using NEBuilder assembly mix resulting in vectors pG3.0-CRISPRoff-v2.1-flbA802, pG3.0-CRISPRoff-v2.1-flbA133, and pG3.0-CRISPRoff-v2.1-glaA (Fig. 1).

Plasmid pG3.0-CRISPRoff-v2.1(-KRAB) was constructed by removing the KRAB domain from plasmid CRISPRoff-v2.1. To this end, CRISPRoff-v2.1 was digested with *PshAI* and *NotI* and self-ligated. The resulting plasmid was cut with *BglII* and *SmaI* to obtain a fragment containing dCas9 and the Dnmt3A-3L domains. This fragment was inserted in *StuI* linearized vector pyrG plug 3.0 (Supplemental Table 1) using NEBuilder® HiFi DNA assembly, resulting in plasmid pG3.0-CRISPRoff-v2.1(-KRAB). The sgRNA sequence targeting the *flbA* promoter 133 bp upstream of the start codon of *flbA* and flanked by the proline tRNA promoter and terminator (see above) was inserted into *PacI* linearized pG3.0-CRISPRoff-v2.1(-KRAB) using NEBuilder assembly mix resulting in vector pG3.0-CRISPRoff-v2.1-flbA133(-KRAB).

For the *flbA* CRISPRon construct (Fig. 2), the TET1-XTEN80-dCas9 gene sequence was cut from plasmid TETv4 (Addgene, #167983; Nuñez et al., 2021) using *EcoRI* and *BglII* and introduced in *StuI* linearized pyrG plug 3.0 vector by using NEBuilder assembly. This was followed by introducing *flbA* sgRNA flanked by the promoter and terminator sequences (see above) in the resulting plasmid pG3.0-TETv4, which had been cut with *PacI* (Fig. 2). This resulted in plasmid pG3.0-TETv4-flbAsgRNA. Targeted gene editing was verified by PCR (Supplemental Fig. 1).

2.3. Inactivation of pyrG in AR9#2

A cytosine deletion at position 378 of the pyrG open reading frame was introduced in strain AR9#2 (Siedenberg et al., 1999) by co-transforming plasmid pFC332-pyrG-sgRNA (consisting of a sgRNA cloned in pFC332 [Addgene plasmid #87845; Nødvig et al., 2015]) and pJET-pyrG (consisting of a PCR-amplified mutant fragment of pyrG cloned in the pJET1.2 backbone [our unpublished data]) (Supplemental Fig. 1). This resulted in strain AR9#2XC (Table 1). Targeted gene editing was verified by PCR (Supplemental Fig. 1).

2.4. Transformation

E.coli TOP10 cells were transformed by heat shock (Froger and Hall, 2007), while *A. niger* was transformed with PEG-mediated protoplast transformation (de Bekker et al., 2009) selecting on MM-SA plates for *pyrG* prototrophy. This was followed by two rounds of purification on MM-G plates and one round on a PDA plate. In order to remove the transforming plasmid, transformants were grown three times on MM-G containing 0.75 mg mL^{-1} 5-fluoroorotic acid (5-FOA) and 10 mM uridine. Transformants that were growing on this medium had lost the plasmid and were auxotrophic for uridine. Plasmid pG3.0-TETv4-flbAsgRNA was introduced in a *flbA* silenced strain again selecting for *pyrG* prototrophy. DNA was extracted from transformants and sequenced by Macrogen (www.macrogen-europe.com) to verify the absence of mutations in the promoter and coding sequence of the target gene.

2.5. RNA extraction and quantitative PCR (qPCR)analysis

RNA was isolated from 3-day-old colonies grown on MM-G between perforated polycarbonate membranes (Wösten et al., 1991). Total RNA was purified using the NucleoSpin® RNA kit (Macherey-NaGEL, Düren, Germany) and reverse transcribed by the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). SYBR Green Q-PCR was used with 1 ng cDNA from biological duplicates and primer pairs FlbA-QPCR-F/FlbA-QPCR-R for *flbA*, GFP-QPCR-F/GFP-QPCR-R for *GFP*, and Actin A-QPCR-F/Actin A-QPCR-R for *actA* that was used as a control (Supplemental Table 3). Expression levels were measured on a ViiA™Real-time PCR system (Applied Biosystems Wilmington DE, USA) using the $2^{-\Delta\Delta CT}$ method for calculation.

2.6. Fluorescence microscopy

Imaging was performed using a Leica MZ16 fluorescence stereomicroscope equipped with a mercury lamp, a Leica GFP2 filter set and a Leica DFC420C digital camera. Fiji 2.1.0 was used for fluorescence intensity analysis.

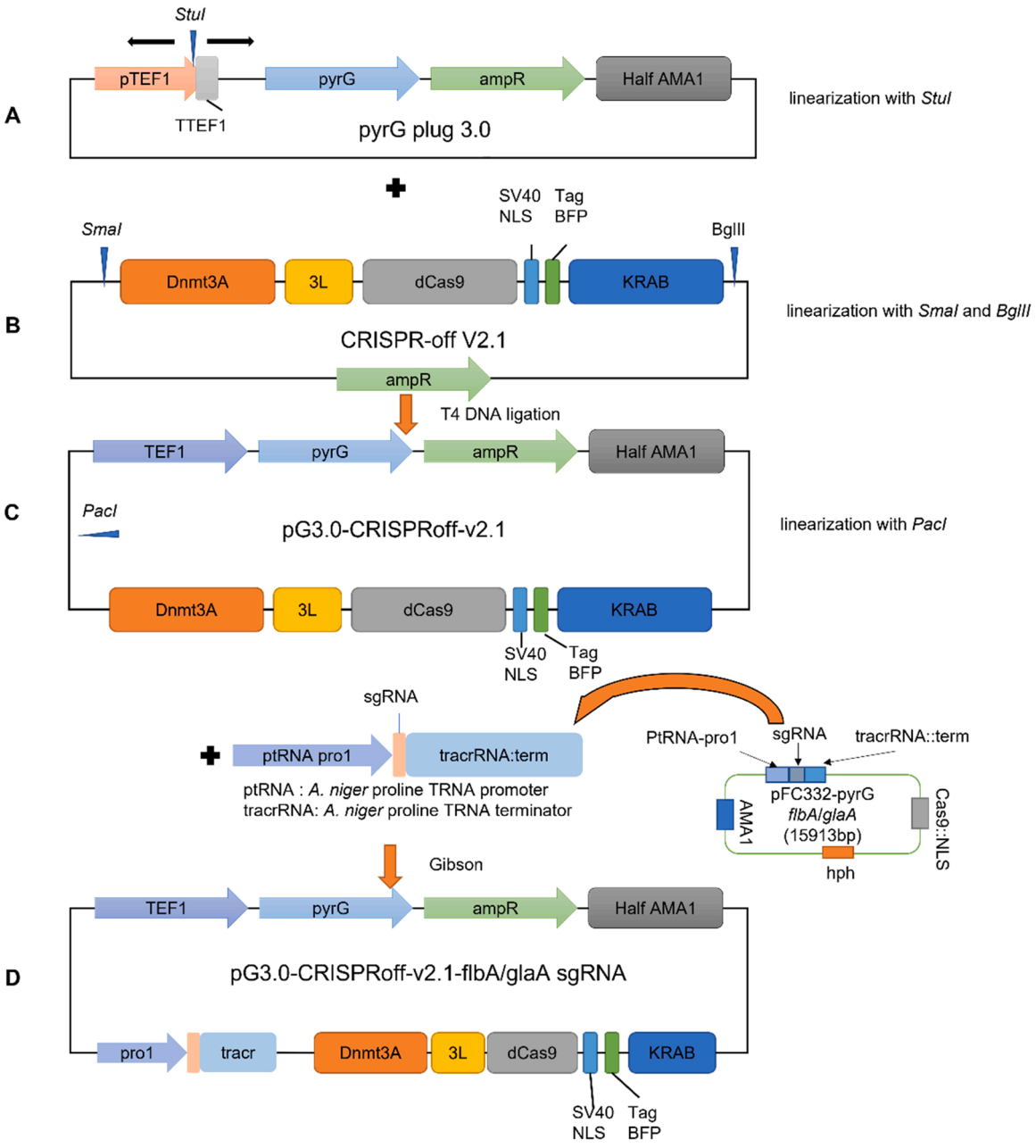

Fig. 1. CRISPRoff constructs of *flbA* and P_{glaA}-*GPF* in *A. niger*. The PflbAS1, PflbAS2, and PglaAS sgRNA's (Supplemental Table 2) target the dCas9 fusion protein to the *flbA* and *glaA* promoter, respectively, thereby silencing the expression of the genes that are under their control. The gene encoding the dCas9 fusion protein was cut from plasmid CRISPRoff V2.1 using *SmaI* and *BglII* (B) and inserted into *StuI* linearized pyrG plug 3.0 (A) resulting in pG3.0-CRISPRoff-v2.1(C). Plasmids pG3.0-CRISPRoff-v2.1-flbA802, pG3.0-CRISPRoff-v2.1-flbA133, and pG3.0-CRISPRoff-v2.1-glaA result from cloning *flbA* and *glaA* sgRNA flanked by their promoter and terminator sequences in plasmid pG3.0-CRISPRoff-v2.1, respectively (D).

2.7. Statistical analysis

All experiments were performed using at least three biological replicates and One-Way Anova (SPSS 25.0) with $p \leq 0.05$.

3. Results

3.1. Applying the CRISPRoff system in A. niger

Plasmids pG3.0-CRISPRoff-v2.1-flbA802, pG3.0-CRISPRoff-v2.1-flbA133 and pG3.0-CRISPRoff-v2.1-glaA were constructed to assess whether the CRISPRoff system V2.1 (Nuñez et al., 2021) works in *A. niger* (Fig. 1). These constructs consist of a gene encoding a catalytically inactive *S. pyogenes* Cas9 protein (dCas9) fused to the ZFN10 KRAB and the D3A and D3L domains. In addition, the plasmids contain a *flbA* or *glaA* sgRNA flanked by proline $tRNA^{pro}$ and $tRNA^{ter}$ sequences. Also, they contain the AMA1_2.8 replication sequence that maintains the construct autonomously in *Aspergillus* as long as there is selection pressure (Sarkari et al., 2017; Aleksenko and Clutterbuck, 1997), which in our system is the presence of the selection gene *pyrG* conferring uridine prototrophy.

The *flbA* constructs that target the dCas9 fusion protein 802 bp (sgRNA-802) or 133 bp (sgRNA-133) upstream of the *flbA* start codon (Fig. 2) were introduced in *A. niger*. Expression and phenotype of *flbA* was assessed in five transformants of each construct (Fig. 3). Four out of five randomly selected sgRNA-802 transformants showed similar or

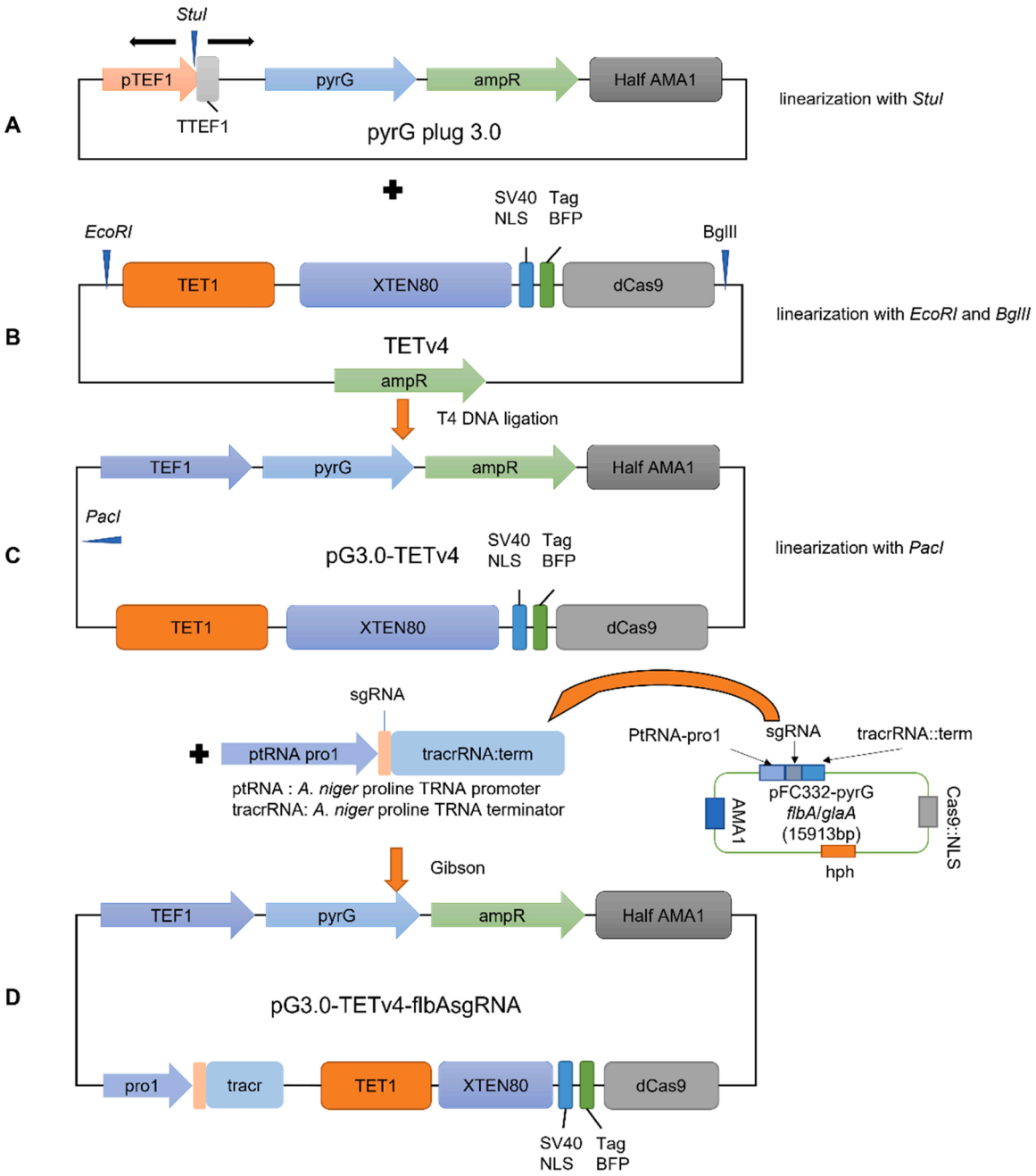

Fig. 2. CRISPRon mediated re-activation of *flbA* in *A. niger*. The gene encoding the fusion protein TET1-XTEN80-dCas9 was cut from plasmid TETv4 using *EcoRI* and *BglII* (B) and inserted into *StuI* digested pyrG plug 3.0 (A), resulting in plasmid pG3.0-TETv4 (C). Plasmid pG3.0-TETv4-flbAsgRNA was constructed by inserting *flbA* sgRNA flanked by their promoter and terminator sequences in plasmid pG3.0-TETv4 (D). This will target TET1-XTEN80-dCas9 to the *flbA* promoter and re-activates expression of *flbA*.

even higher *flbA* expression when compared to wild-type, while transformant CFFA1–2 showed 80 % reduced expression of the gene. In contrast, sgRNA-133 transformants all showed reduced *flbA* expression with transformant CFFA2–8 even showing 90 % reduced expression. The phenotype of the transformants correlated with the reduced *flbA* expression. Strain CFFA2–8 formed the least number of spores of the transformants but sporulation was not completely abolished as in strain Δ*flbA* (Fig. 3).

Strain *A. niger* AR9#2 expresses *GFP* from the *glaA* promoter. The derivative of this strain, called AR9#2XC, in which *pyrG* is inactivated, was transformed with a construct targeting the dCas9 fusion protein 129 bp upstream from the start codon of *GFP*. This site was chosen because sgRNA-133 showed higher decreased *flbA* silencing when compared to sgRNA-802. Expression of *GFP* was reduced 3–10 fold in three out of four randomly picked transformants when compared to AR9#2XC, while expression in one transformant was not affected (Fig. 4). Also in this case, qPCR results correlated with GFP fluorescence.

Next, it was assessed whether the phenotype of *flbA* transformants can become stronger by transferring 10 randomly picked transformants 10 times on a fresh selection medium. A total of five transformants indeed showed a stronger *flbA* phenotype (data not shown). Stability of gene silencing of *flbA* was tested in strain CFFA2–8 that shows a strong reduction in *flbA* expression (Fig. 3). To this end, the CRISPRoff plasmid pG3.0-CRISPRoff-v2.1-flbA133 was removed from the strain by growing three times on a medium with uridine (thus removing selection pressure) as well as 5-FOA to select for *pyrG* auxotrophic strains. Eight

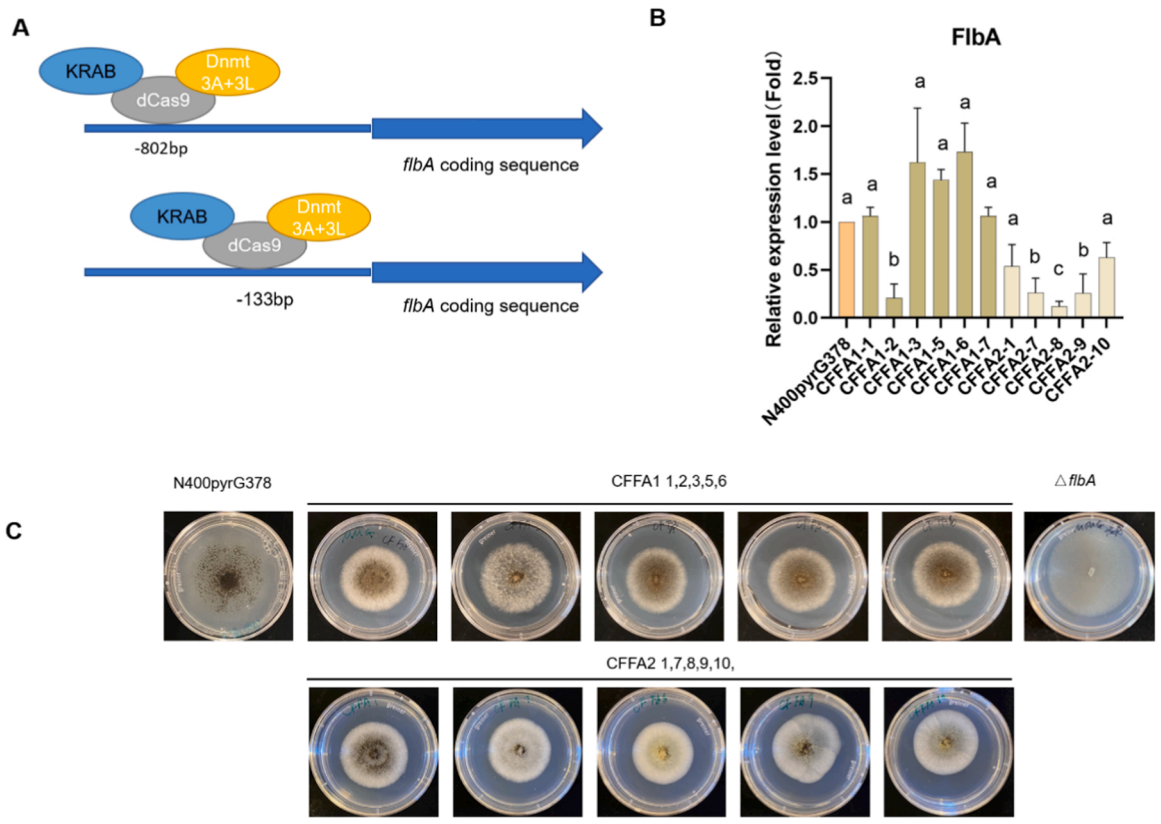

Fig. 3. Transcriptional repression of *flbA* using the CRISPRoff system. The sgRNA's targeted the dCas9 fusion protein 802 bp (transformants CFFA1-1-6) or 133 bp (transformants CFFA2–1–10) upstream of the start codon (A). Expression level of *flbA* as shown by RT-QPCR (B) and phenotypes (C) of 3-day-old and 5-day-old-colonies of transformants, respectively. The non-silenced N400pyrG378 strain was used as a control for expression studies, while this strain as well as strain Δ*flbA* were used as controls for phenotyping. Gene *actA* was used to normalize *flbA* expression. Error bars represent standard error of the mean of three biological replicates. Statistical analysis was done using One-Way ANOVA with different letters indicating statistical difference.

randomly picked colonies were streaked on a medium with uridine resulting in strains that had lost the CRISPRoff plasmid and, as expected, that could not grow on a medium without uridine. Seven of the resulting strains still showed the original CFFA2–8 phenotype, while one of the eight strains, called CFFA2–8–1, showed the same phenotype as the Δ*flbA* strain that produces a yellow pigment, that did not sporulate (Fig. 5) and that showed undetectable *flbA* expression (Fig. 6BC).

3.2. Confirmation of DNA methylation in A. niger

To verify DNA methylation after introducing the CRISPRoff system, *A. niger* was transformed with the *flbA* CRISPRoff plasmid pG3.0-CRISPRoff-v2.1-flbAsgRNA133, plasmid pG3.0-CRISPRoff-v2.1 that lacks the *flbA* sgRNA, or plasmid pG3.0-CRISPRoff-flbAsgRNA133 (-KRAB) that contains the *flbA* sgRNA but lacks the KRAB domain. As mentioned above introducing the *flbA* CRISPRoff plasmid pG3.0-CRISPRoff-v2.1-flbAsgRNA133 resulted in reduced sporulation (Fig. 7A). Similarly, reduced sporulation was obtained in transformants in which the plasmid lacking the KRAB domain was introduced. In contrast, no reduction in sporulation was obtained when the sgRNA was lacking. Taken together, the sgRNA and the D3A and D3L domains are needed for a Δ*flbA*-like phenotype. A DNA restriction / PCR strategy was performed to confirm that the D3A and D3L domains result in methylation of the promoter region of *flbA*. To this end, genomic DNA of the non-silenced N400pyrG378 strain, the CRISPRoff transformants CFFA1–1, CFFA1–2, CFFA1–3, CFFA2–1 and CFFA2–2, as well as the CRISPRon transformants TEFA1–1 and TEFA1–2 was digested with *Bsu15I*. This restriction enzyme is sensitive to CpG DNA methylation. After *Bsu15I* incubation, a PCR was performed using primers flanking the restriction site in the *flbA* promoter (Fig. 7BC; Supplemental Table 3). Genomic DNA of the non-silenced N400pyrG378 strain showed, as expected, a PCR fragment without restriction but no fragment with restriction. Similar results were obtained in the CRISPRon strains TEFA1–1 and TEFA1–2. In contrast, the CRISPRoff strains CFFA1–1, CFFA1–2, CFFA1–3, CFFA2–1 and CFFA2–2 resulted in PCR fragments in both conditions. Together, these results indicate that the DNA in the *flbA* promoter region is methylated. The same results were obtained with the CFFA1–1, CFFA1–2, CFFA1–3, CFFA2–1 and CFFA2–2 strains, after they had been restreaked to MM-G medium. This shows that the methylation is stable in the presence of the *flbA* CRISPRoff plasmid pG3.0-CRISPRoff-v2.1-flbAsgRNA133 (data not shown).

3.3. Impact of location of sgRNA targeting site

The impact of the location of the sgRNA targeting site was assessed. To this end, sgRNA's were designed localizing to 10 different positions in the *flbA* gene ranging between −802 (sgRNA-802) and +861 (sgRNA+861) bp from its start codon (Fig. 8). These sgRNAs were cloned in plasmid pG3.0-CRISPRoff-v2.1 and introduced in strain N400pyrG378. RNA level of *flbA* was quantified by RT-qPCR of 3-day-old colonies of four randomly picked transformants of each sgRNA targeting site. Strain N400pyrG378 containing plasmid pG3.0-CRISPRoff-v2.1 (without sgRNA) was used as a control. All sgRNAs resulted in decreased *flbA* expression. The lowest average expression levels in transformants were obtained with sgRNA-731, sgRNA+670, and sgRNA-133 with mRNA levels of 27 %, 37 % and 42 % compared to that of the control, respectively. Together, results indicate that there is not a preferred location for the sgRNA targeting site of the CRISPRoff system relating to the distance of the *flbA* start codon.

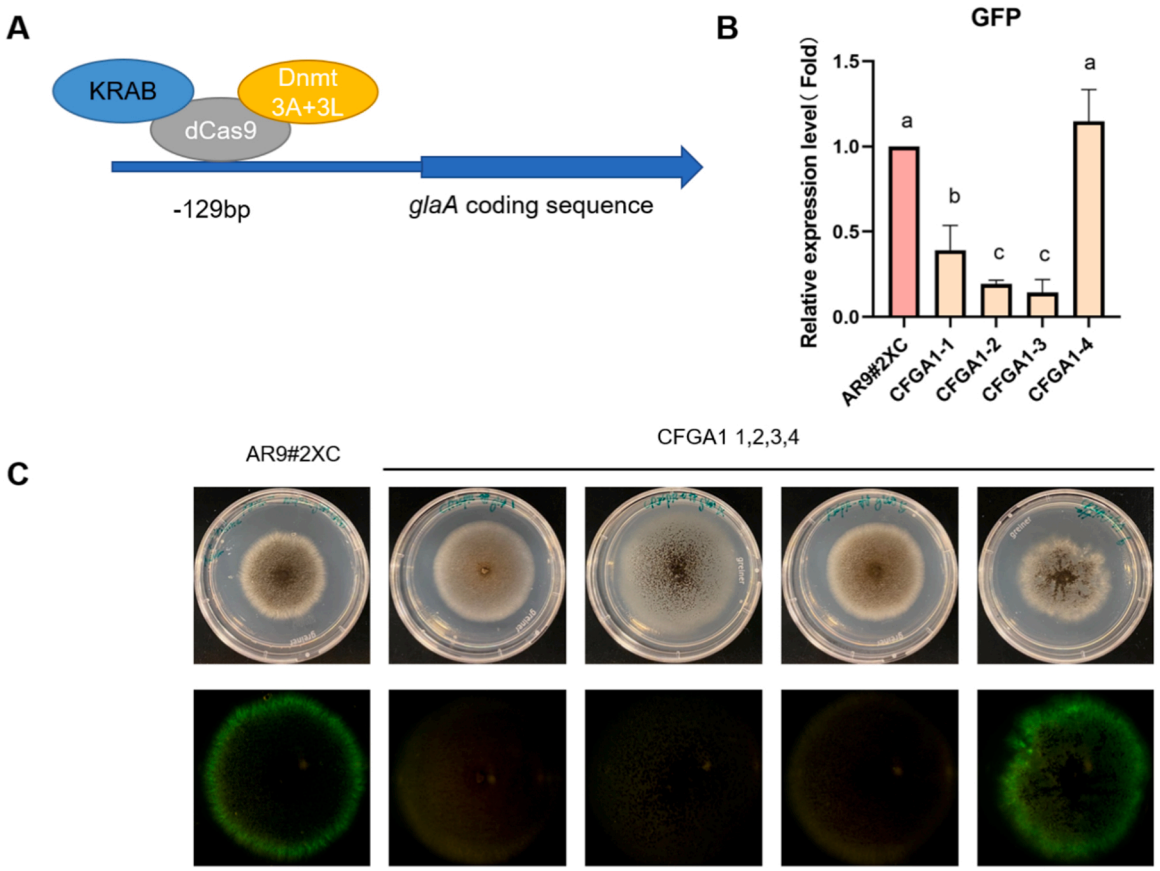

Fig. 4. Transcriptional repression of *GFP* using the CRISPRoff system. The sgRNA targeted the dCas9 fusion protein 129 bp upstream of the start codon in the *glaA* promoter (A). Expression level of *GFP* as shown by RT-QPCR (B) and phenotypes (C) of 3-day-old and 5-day-old-colonies, respectively, of transformants and the parental strain AR9#2XC that was used as a control. The *actA* gene was used to normalize *flbA* expression. Error bars represent standard error of the mean of three biological replicates. Statistical analysis was done using One-Way ANOVA with different letters indicating statistical difference.

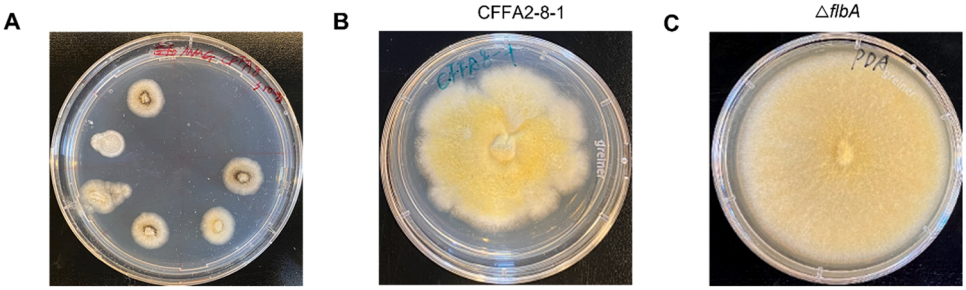

Fig. 5. Stability of gene silencing after removing the CRISPRoff plasmid from transformant CFFA2–8. This strain was grown three times on a medium containing 5-FOA and uridine (A). The phenotype of one of the resulting strains, CFFA2–8–1 (B) is even similar to that of Δ*flbA* (C) showing the stability of gene silencing and the phenomenon of increasing phenotype by re-streaking.

3.4. Applying CRISPRon system to Aspergillus niger

Strain CFFA2-8-1 (see above) was transformed with pG3.0-TETv4-flbAsgRNA (Fig. 2) to assess whether *flbA* expression can be re-activated by the CRISPRon system. Indeed, 8 transformants (TEFA 1–8) showed full sporulation and *flbA* expression 50–150 % of the wild-type (Fig. 6). Together, the CRISPRon system can be used to revert gene silencing of the CRISPRoff system in *A. niger*.

4. Discussion

Protein dCas9 that is targeted by a sgRNA can inhibit gene expression due to its DNA binding in a promoter of a gene (Nuñez et al., 2021). Gene expression can also be inhibited by guiding dCas9 to the open reading frame of a gene as a result of hampered RNA polymerase activity (Larson et al., 2013; Qi et al., 2013). Protein dCas9 can also be fused to proteins or protein domains to regulate gene expression. For instance, dCas9 has been fused to transcription activators and repressors in human cells (Alerasool et al., 2020), plants (Vazquez-Vilar et al., 2021) and microbes like *Myxococcus xanthus* (Peng et al., 2018), *Aspergillus nidulans* (Schüller et al., 2020), *A. niger* (Yu et al., 2022) and *Pichia pastoris* (Liao et al., 2021). In the latter case, dCas9 was fused with the transcriptional repressor Mxi1/RD1152 or the transcriptional activator VPR. This resulted in a 70 % inhibition and up to ~3.5-fold activation, respectively. Protein dCas9 has also been fused to epigenetic regulators to modulate expression in human cells (Hilton et al., 2015; Amabile et al.,

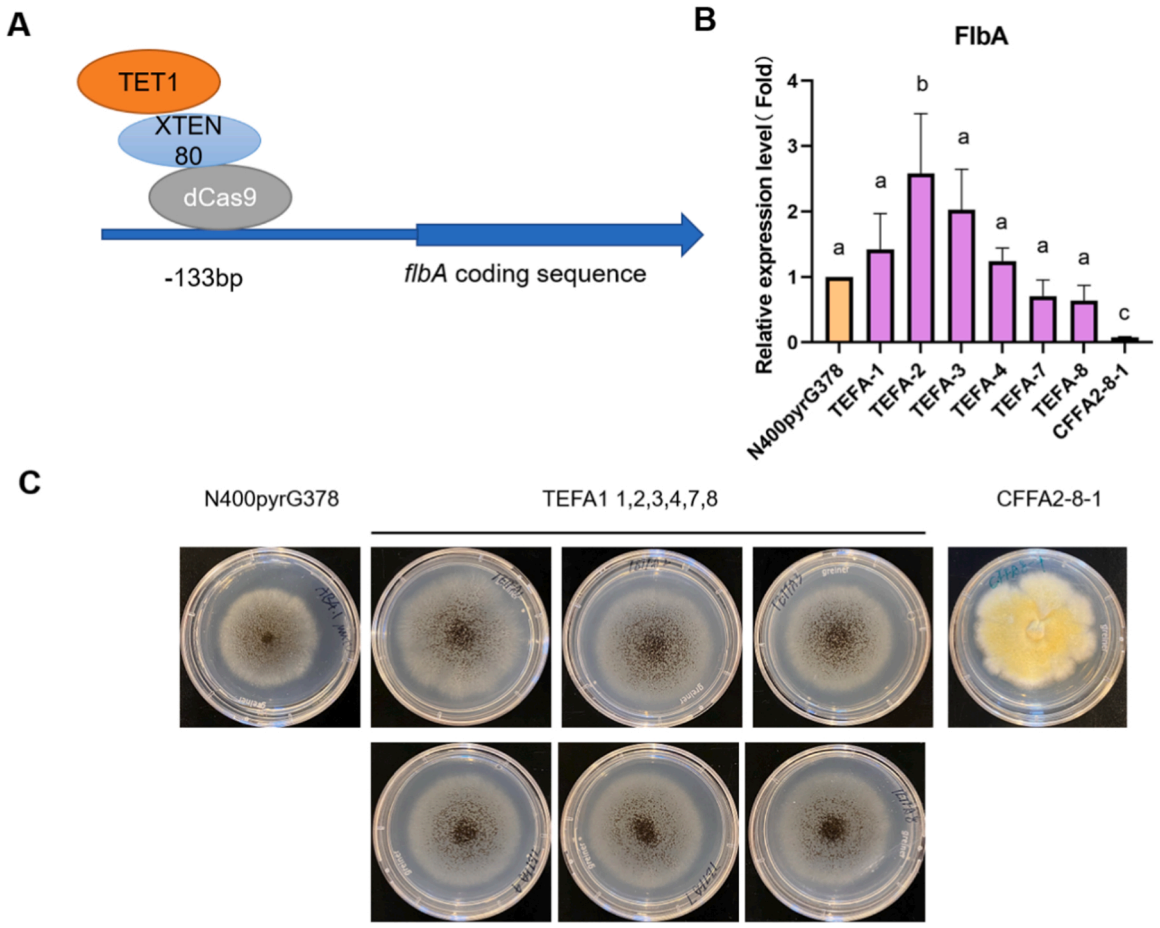

Fig. 6. Transcriptional re-activation of *flbA* in strain CFFA2–8–1 using the CRISPRon system. Schematic representation of the targeting of the TET1-XTEN80-dCas9 fusion protein to the promoter of *flbA* (A). Expression level of *flbA* as shown by RT-QPCR (B) and phenotypes (C) of 3-day-old and 5-day-old-colonies, respectively, of transformants TEFA1–8 and host strain CFFA2–8–1. Strain N400pyrG378 was used as a control in the expression analysis, while CFFA2–8–1 was used as a control for phenotyping. The *actA* gene was used to normalize *flbA* expression. Error bars represent standard error of the mean of three biological replicates. Different letters indicate statistical difference.

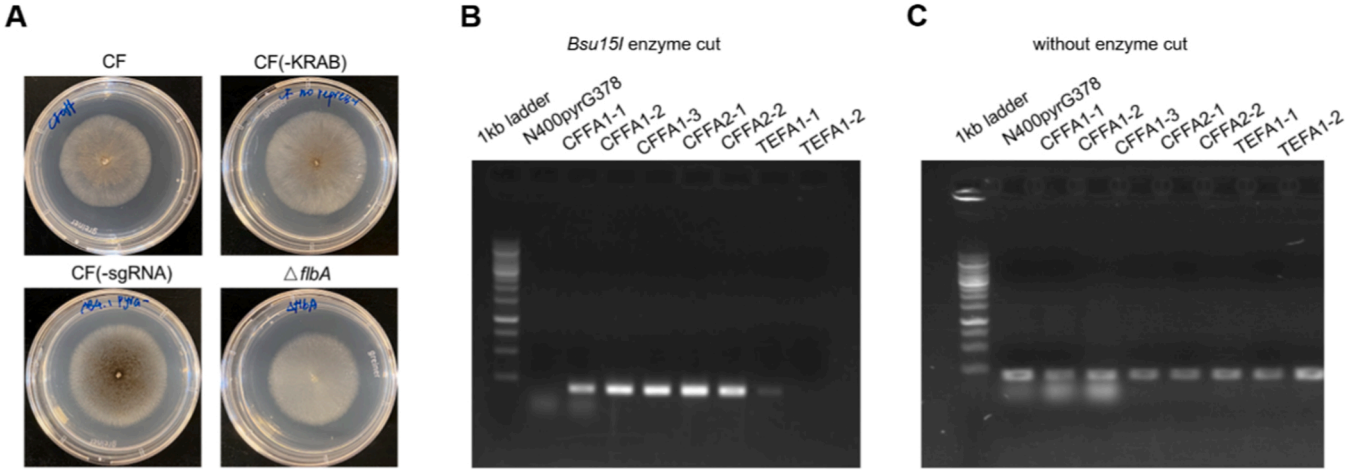

Fig. 7. Phenotype of *flbA* CRISPRoff (CF), *flbA* CRISPRoff(-KRAB) (CF(-KRAB), CRISPRoff (CF-(sgRNA)), and △*flbA* (A) and PCR verification of methylation with (B) and without (C) *Bsu15I* restriction of genomic DNA before PCR amplification. Genomic DNA of the non-silenced N400pyrG378 strain shows as expected a PCR fragment without restriction but no fragment with restriction. Similar results are obtained in the CRISPRon strains TEFA1–1 and TEFA1–2. In contrast, the CRISPRoff strains CFFA1–1, CFFA1–2, CFFA1–3, CFFA2–1 and CFFA2–2 result in PCR fragments in both conditions because the restriction enzyme cannot cut in methylated DNA.

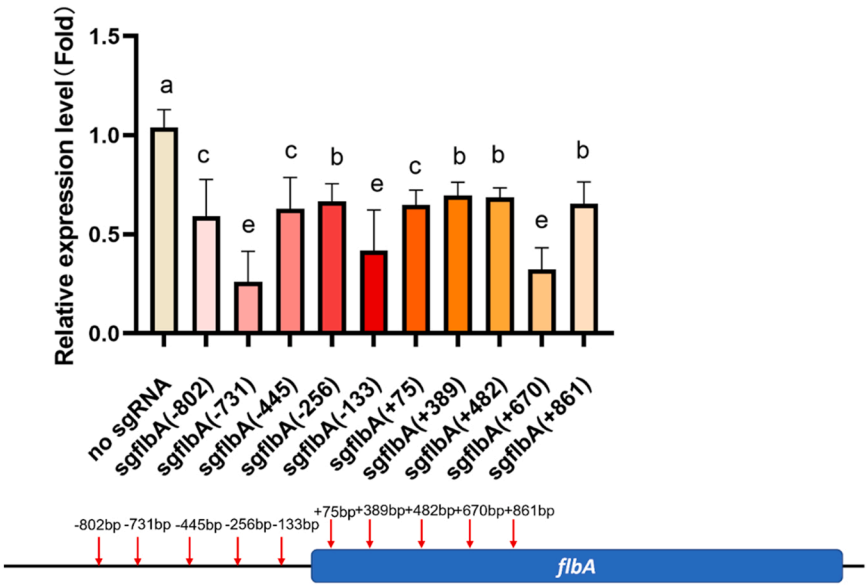

Fig. 8. Impact of sgRNA localization on *flbA* expression. sgRNA's were targeted to 10 different positions ranging between −802 (sgRNA-802) and +861 (sgRNA+861) bp from the start codon of *flbA*. RNA level of *flbA* was quantified by RT-qPCR of 3-day-old colonies. Strain N400pyrG378 in which plasmid pG3.0-CRISPRoff-v2.1 (without sgRNA) was introduced was used as a control. The *actA* gene was used to normalize *flbA* expression. Error bars represent standard error of the mean of three biological replicates. Statistical analysis was done using One-Way ANOVA with different letters indicating statistical difference.

2016), *Plasmodium falciparum* (Xiao et al., 2019) and *A. niger* (Li et al., 2021; Yu et al., 2022). For instance, a gene fusion of the histone acetylase P300 and dCas9 increased *breF* expression in *A. niger* 3-fold (Li et al., 2021). Here, we successfully used the CRISPRoff and CRISPRon systems for the first time in a fungus. These systems were developed to silence genes in human stem cells resulting from targeted epigenetic changes and to revert these changes, respectively (Nuñez et al., 2021).

We used the CRISPRoff system to reduce expression of *flbA* and *GFP*. Some transformants showed undetectable GFP fluorescence (data not shown) or undetectable *flbA* mRNA levels after introducing the CRISPRoff system, while others showed an intermediate phenotype. Apparently, efficiency of silencing is variable between transformants. This may be caused by instability of the AMA1 plasmid. Nuclei contain 10 of such plasmids per nucleus and the expression of genes contained on the vector is proportional to copy number (Aleksenko et al., 1996). However, the stability of the plasmids is limited even under selective conditions (Aleksenko and Clutterbuck, 1997). In fact, nuclei can be totally devoid of the plasmid. Hyphae or compartments having such nuclei would survive their auxotrophy by flow of nutrients from hyphae that do have the AMA1 plasmid and are therefore prototrophic (in our case for uridine).

Silencing of *flbA* was maintained after removing the CRISPRoff plasmid, thus showing that the changes are inherited in the fungal system. What is the evidence that silencing is caused by methylation? First, the *flbA* CRISPRoff phenotype was reversed by introducing the CRISPRon system. Second, removing the KRAB domain from the CRISPRoff plasmid still reduced *flbA* expression as shown by reduced sporulation of the colony. In contrast, removing the sgRNA from the CRISPR construct did not result in reduced sporulation. Together, the sgRNA and the D3A and D3L domains are needed for reducing *flbA* expression. Finally, a DNA restriction / PCR strategy confirmed that the D3A and D3L domains result in methylation of the promoter region of *flbA*. This was shown by the fact that the methylation sensitive *Bsu15I* enzyme did cut in the *flbA* promoter of non-silenced strains but did not cut in the CRISPRoff strains. Together, these results strongly indicate that the DNA in the *flbA* promoter region is methylated. Notably, methylation is maintained, at least partially, after removing the plasmid containing the D3A and D3L domains. This implies that *A. niger* has DNA methylation activity. Very little methylation has been reported in *Aspergillus flavus* (Gowher et al., 2001) but methylation has not been shown in *Aspergillus nidulans* and *A. niger* (Tamame et al., 1983; Liu et al., 2012; Lee et al., 2008). Future research should assess the methylation maintenance system in *A. niger*.

A screen of 41 genes showed that efficient gene repression is obtained in human cells when sgRNAs are targeted −50 bp to +250 bp relative to the transcription start site (TSS) (Radzisheuskaya et al., 2016). Similarly, gene expression in *S. cerevisiae* is most affected when the sgRNA is targeted in the 200 bp region upstream of the TSS (Smith et al., 2016; McGlincy et al., 2021). We here targeted the dCas9 fusion protein of the CRISPRoff system to five locations in the *flbA* promoter and to five locations within its coding sequence. All sgRNAs effectively decreased *flbA* expression targeting positions within the −802 bp to +861 bp region relative to the start codon. The lowest expression levels were obtained far upstream (−731 bp) and downstream (+670 bp) of the start codon (i.e. −664 and +737 bp of the TSS) with average expression levels in transformants of 27–37 % when compared to the control. These results indicate that there is not a preferred location for the sgRNA targeting site of the CRISPRoff system in *A. niger*. It should be assessed in the future whether this is also the case for other genes of this fungus.

Together, the CRISPRoff and CRISPRon system can be used to modulate gene expression without the risk of genome rearrangements due to the double strand breaks that are introduced by Cas9. Future research will assess whether the CRISPRoff and CRISPRon systems can be optimized. Literature suggests that this for instance can be achieved by using codon optimized versions of the domains, replacing the promoter that controls expression of the sgRNA, replacing the ZNF10 KRAB domain by an orthologous domain of another protein, or by replacing the KRAB domain for the MxiI domain (Gilbert et al., 2013; Schwartz et al., 2017; Wensing et al., 2019; Zheng et al., 2019; Alerasool et al., 2020; Cámara et al., 2020; McGlincy et al., 2021; Wang et al., 2021; Replogle et al., 2022; Yu et al., 2022). Such optimizations should result in a high incidence of strong reduction in gene expression in the case of the CRISPRoff system and in high incidence of full recovery of gene expression in the case of the CRISPRon system.

CRediT authorship contribution statement

XC: Conceptualization; Formal analysis; Investigation; Methodology; Writing - original draft. JPMT: Conceptualization; Methodology. YL: Investigation. LGL: Supervision; HABW: Supervision; Writing - review & editing.

Declaration of Competing Interest

The authors do not have a declaration of interest.

Data availability

Data will be made available on request.

Acknowledgments

XC is supported by a grant of the China Scholarship Council CSC.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127397.

References

Aleksenko, A., Clutterbuck, A.J., 1997. Autonomous plasmid replication in *Aspergillus nidulans*: AMA1 and MATE elements. Fungal Genet. Biol. 21, 373–387. https://doi.org/10.1006/fgbi.1997.0980.

Aleksenko, A., Nikolaev, I., Vinetski, Y., Clutterbuck, A.J., 1996. Gene expression from replicating plasmids in *Aspergillus nidulans*. Mol. Gen. Genet. 253, 242–246. https://doi.org/10.1007/s004380050318.

Alerasool, N., Segal, D., Lee, H., Taipale, M., 2020. An efficient KRAB domain for CRISPRi applications in human cells. Nat. Methods 17, 1093–1096. https://doi.org/10.1038/s41592-020-0966-x.

Amabile, A., Migliara, A., Capasso, P., Biffi, M., Cittaro, D., Naldini, L., Lombardo, A., 2016. Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. Cell 167, 219–232. https://doi.org/10.1016/j.cell.2016.09.006.

Cámara, E., Lenitz, I., Nygård, Y.A., 2020. CRISPR activation and interference toolkit for industrial *Saccharomyces cerevisiae* strain KE6-12. Sci. Rep. 10, 14605. https://doi.org/10.1038/s41598-020-71648-w.

Cullot, G., Boutin, J., Toutain, J., Prat, F., Pennamen, P., Rooryck, C., Teichmann, M., Rousseau, E., Lamrissi-Garcia, I., Guyonnet-Duperat, V., Bibeyran, A., Lalanne, M., Prouzet-Mauléon, V., Turcq, B., Ged, C., Blouin, J.M., Richard, E., Dabernat, S., Moreau-Gaudry, F., Bedel, A., 2019. CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. Nat. Commun. 10, 1136. https://doi.org/10.1038/s41467-019-09006-2.

de Bekker, C., Wiebenga, L.A., Aguilar, G., Wösten, H.A.B., 2009. An enzyme cocktail for efficient protoplast formation in *Aspergillus niger*. J. Microbiol. Meth. 76, 305–306. https://doi.org/10.1016/j.mimet.2008.11.001.

Froger, A., Hall, J.E., 2007. Transformation of plasmid DNA into *E. coli* using the heat shock method. J. Vis. Exp. 6, 253. https://doi.org/10.3791/253.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442–451. https://doi.org/10.1016/j.cell.2013.06.044.

Gowher, H., Ehrlich, K.C., Jeltsch, A., 2001. DNA from *Aspergillus flavus* contains 5-methylcytosine. FEMS Microbiol. Lett. 205, 151–155. https://doi.org/10.1111/j.1574-6968.2001.tb10939.x.

Hilton, I.B., D'Ippolito, A.M., Vockley, C.M., Thakore, P.I., Crawford, G.E., Reddy, T.E., Gersbach, C.A., 2015. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat. Biotechnol. 33, 510–517. https://doi.org/10.1038/nbt.3199.

Holtzman, L., Gersbach, C.A., 2018. Editing the epigenome: reshaping the genomic landscape. Ann. Rev. Genom. Hum. Genet. 19, 43–71. https://doi.org/10.1146/annurev-genom-083117-021632.

Krijgsheld, P., Nitsche, B.M., Post, H., Levin, A.M., Müller, W.H., Heck, A.J., Ram, A.F., Altelaar, A.F., Wösten, H.A.B., 2013. Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. J. Proteome Res 12, 1808–1819. https://doi.org/10.1021/pr301154w.

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat. Protoc. 8, 2180–2196. https://doi.org/10.1038/nprot.2013.132.

Lee, D.W., Freitag, M., Selker, E.U., Aramayo, R., 2008. A cytosine methyltransferase homologue is essential for sexual development in *Aspergillus nidulans*. PLoS ONE 3, e2531. https://doi.org/10.1371/journal.pone.0002531.

Li, X., Huang, L., Pan, L., Wang, B., Pan, L., 2021. CRISPR/dCas9-mediated epigenetic modification reveals differential regulation of histone acetylation on *Aspergillus niger* secondary metabolite. Microbiol. Res. 245, 126694 https://doi.org/10.1016/j.micres.2020.126694.

Liao, X., Li, L., Jameel, A., Xing, X.H., Zhang, C., 2021. A versatile toolbox for CRISPR-based genome engineering in *Pichia pastoris*. Appl. Microbiol. Biotechnol. 105, 9211–9218. https://doi.org/10.1007/s00253-021-11688-y.

Liu, S.Y., Lin, J.Q., Wu, H.L., Wang, C.C., Huang, S.J., Luo, Y.F., Sun, J.H., Zhou, J.X., Yan, S.J., He, J.G., Wang, J., He, Z.M., 2012. Bisulfite sequencing reveals that *Aspergillus flavus* holds a hollow in DNA methylation. PLoS One 7, e30349. https://doi.org/10.1371/journal.pone.0030349.

Lupo, A., Cesaro, E., Montano, G., Zurlo, D., Izzo, P., Costanzo, P., 2013. KRAB-zinc finger proteins: a repressor family displaying multiple biological functions. Curr. Genom. 14, 268–278. https://doi.org/10.2174/1389202911314990002.

Maeder, M.L., Angstman, J.F., Richardson, M.E., Linder, S.J., Cascio, V.M., Tsai, S.Q., Ho, Q.H., Sander, J.D., Reyon, D., Bernstein, B.E., Costello, J.F., Wilkinson, M.F., Joung, J.K., 2013. Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. Nat. Biotechnol. 31, 1137–1142. https://doi.org/10.1038/nbt.2726.

McGlincy, N.J., Meacham, Z.A., Reynaud, K.K., Muller, R., Baum, R., Ingolia, N.T., 2021. A genome-scale CRISPR interference guide library enables comprehensive phenotypic profiling in yeast. BMC Genom. 22, 205. https://doi.org/10.1186/s12864-021-07518-0.

Nødvig, C.S., Nielsen, J.B., Kogle, M.E., Mortensen, U.H., 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS ONE 10, e0133085. https://doi.org/10.1371/journal.pone.0133085.

Nuñez, J.K., Chen, J., Pommier, G.C., Cogan, J.Z., Replogle, J.M., Adriaens, C., Ramadoss, G.N., Shi, Q., Hung, K.L., Samelson, A.J., Pogson, A.N., Kim, J.Y.S., Chung, A., Leonetti, M.D., Chang, H.Y., Kampmann, M., Bernstein, B.E., Hovestadt, V., Gilbert, L.A., Weissman, J.S., 2021. Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. Cell 184, 2503–2519. https://doi.org/10.1016/j.cell.2021.03.025.

O'Geen, H., Bates, S.L., Carter, S.S., Nisson, K.A., Halmai, J., Fink, K.D., Rhie, S.K., Farnham, P.J., Segal, D.J., 2019. Ezh2-dCas9 and KRAB-dCas9 enable engineering of epigenetic memory in a context-dependent manner. Epigenetics Chromatin 12, 26. https://doi.org/10.1186/s13072-019-0275-8.

Peng, R., Wang, Y., Feng, W.W., Yue, X.J., Chen, J.H., Hu, X.Z., Li, Z.F., Sheng, D.H., Zhang, Y.M., Li, Y.Z., 2018. CRISPR/dCas9-mediated transcriptional improvement of the biosynthetic gene cluster for the epothilone production in *Myxococcus xanthus*. Microb. Cell Fact. 17, 1–12. https://doi.org/10.1186/s12934-018-0867-1.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022.

Radzisheuskaya, A., Shlyueva, D., Müller, I., Helin, K., 2016. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. Nucl. Acids Res. 44, e141 https://doi.org/10.1093/nar/gkw583.

Replogle, J.M., Bonnar, J.L., Pogson, A.N., Liem, C.R., Maier, N.K., Ding, Y., Russell, B.J., Wang, X., Leng, K., Guna, A., Norman, T.M., Pak, R.A., Ramos, D.M., Ward, M.E., Gilbert, L.A., Kampmann, M., Weissman, J.S., Jost, M., 2022. Maximizing CRISPRi efficacy and accessibility with dual-sgRNA libraries and optimal effectors. Elife 11, e81856. https://doi.org/10.7554/eLife.81856.

Sarkari, P., Marx, H., Blumhoff, M.L., Mattanovich, D., Sauer, M., Steiger, M.G., 2017. An efficient tool for metabolic pathway construction and gene integration for *Aspergillus niger*. Bioresour. Technol. 245, 1327–1333. https://doi.org/10.1016/j.biortech.2017.05.004.

Schüller, A., Wolansky, L., Berger, H., Studt, L., Gacek-Matthews, A., Sulyok, M., Strauss, J.A., 2020. Novel fungal gene regulation system based on inducible VPR-dCas9 and nucleosome map-guided sgRNA positioning. Appl. Microbiol. Biotechnol. 104, 9801–9822. https://doi.org/10.1007/s00253-020-10900-9.

Schwartz, C., Frogue, K., Ramesh, A., Misa, J., Wheeldon, I., 2017. CRISPRi repression of nonhomologous end-joining for enhanced genome engineering via homologous recombination in *Yarrowia lipolytica*. Biotechnol. Bioeng. 114, 2896–2906. https://doi.org/10.1002/bit.26404.

Siedenberg, D., Mestric, S., Ganzlin, M., Schmidt, M., Punt, P.J., van den Hondel, C.A.M.J.J., Rinas, U., 1999. GlaA promoter controlled production of a mutant green fluorescent protein (S65T) by recombinant *Aspergillus niger* during growth on defined medium in batch and fed-batch cultures. Biotechnol. Prog. 15, 43–50. https://doi.org/10.1021/bp980105u.

Smith, J.D., Suresh, S., Schlecht, U., Wu, M., Wagih, O., Peltz, G., Davis, R.W., Steinmetz, L.M., Parts St, L., Onge, R.P., 2016. Quantitative CRISPR interference screens in yeast identify chemical-genetic interactions and new rules for guide RNA design. Genome Biol. 17, 45. https://doi.org/10.1186/s13059-016-0900-9.

Tamame, M., Antequera, F., Villanueva, J.R., Santos, T., 1983. High-frequency conversion to a "fluffy" developmental phenotype in *Aspergillus* spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. Mol. Cell Biol. 3, 2287–2297. https://doi.org/10.1128/mcb.3.12.2287-2297.

Urrutia, R., 2003. KRAB-containing zinc-finger repressor proteins. Genome Biol. 4, 231. https://doi.org/10.1186/gb-2003-4-10-231.

Vazquez-Vilar, M., Garcia-Carpintero, V., Selma, S., Bernabé-Orts, J.M., Sanchez-Vicente, J., Salazar-Sarasua, B., Ressa, A., de Paola, C., Ajenjo, M., Quintela, J.C., Fernández-Del-Carmen, A., Granell, A., Orzáez, D., 2021. The GB4.0 platform, an all-in-one tool for CRISPR/Cas-Based multiplex genome engineering in plants. Front. Plant Sci. 12, 689937 https://doi.org/10.3389/fpls.2021.689937.

Vishniac, W., Santer, M., 1957. The thiobacilli. Bacteriol. Rev. 21, 195–213. https://doi.org/10.1128/br.21.3.195-213.1957.

Wang, Q., Zhao, Q., Liu, Q., He, X., Zhong, Y., Qin, Y., Gao, L., Liu, G., Qu, Y., 2021. CRISPR/Cas9-mediated genome editing in *Penicillium oxalicum* and *Trichoderma reesei* using 5S rRNA promoter-driven guide RNAs. Biotechnol. Lett. 43, 495–502. https://doi.org/10.1007/s10529-020-03024-7.

Wensing, L., Sharma, J., Uthayakumar, D., Proteau, Y., Chavez, A., Shapiro, R.S., 2019. A CRISPR interference platform for efficient genetic repression in *Candida albicans*. mSphere 4, e00002–e00019. https://doi.org/10.1128/mSphere.00002-19.

Wieser, J., Lee, B.N., Fondon 3rd, J.W., Adams, T.H., 1994. Genetic requirements for initiating asexual development in *Aspergillus nidulans*. Curr. Genet. 27, 62–69. https://doi.org/10.1007/BF00326580.

Wösten, H.A.B., Moukha, S.M., Sietsma, J.H., Wessels, J.G.H., 1991. Localization of growth and secretion of proteins in *Aspergillus niger*. J. Gen. Microbiol. 137, 2017–2023. https://doi.org/10.1099/00221287-137-8-2017.

Xiao, B., Yin, S., Hu, Y., Sun, M., Wei, J., Huang, Z., Wen, Y., Dai, X., Chen, H., Mu, J., Cui, L., Jiang, L., 2019. Epigenetic editing by CRISPR/dCas9 in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U. S. A. 116, 255–260. https://doi.org/10.1073/pnas.1813542116.

Xu, X., Tao, Y., Gao, X., Zhang, L., Li, X., Zou, W., Ruan, K., Wang, F., Xu, G.L., Hu, R., 2016. A CRISPR-based approach for targeted DNA demethylation. Cell Discov. 2, 16009. https://doi.org/10.1038/celldisc.2016.9.

Yu, J.H., Wieser, J., Adams, T.H., 1996. The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J. 15, 5184–5290. https://doi.org/10.1002/j.1460-2075.1996.tb00903.x.

Yu, L.Y., Li, L.X., Yao, L.L., Zheng, J.W., Wang, B., Pan, L., 2022. A special phenotype of aconidial *Aspergillus niger* SH2 and its mechanism of formation via CRISPRi. J. Fungi 8, 679. https://doi.org/10.3390/jof8070679.

Zheng, X., Zheng, P., Zhang, K., Cairns, T.C., Meyer, V., Sun, J., Ma, Y., 2019. 5S rRNA promoter for guide RNA expression enabled highly efficient CRISPR/Cas9 genome editing in *Aspergillus niger*. ACS Synth. Biol. 8, 1568–1574. https://doi.org/10.1021/acssynbio.7b00456.