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ORIGINAL ARTICLE

Optimization of the Genome Editing CRISPR-Cas9 Technology in *Scedosporium apiospermum*

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Abstract *Scedosporium* species are opportunistic pathogens causing a large variety of human infections. To date, there is limited information on the pathogenic mechanisms of these fungi, partly because of the limited number of genetic tools available. Here, the CRISPR-Cas9 technology, which provided promising results for functional genomic studies in filamentous fungi, was optimized for *Scedosporium* species using in vitro assembled Cas9 ribonucleoprotein (RNP) complexes. In these fungi, functional genomic studies are particularly complex in a wild-type strain, because of the high frequency of non-homologous recombination. Prior disruption of the *KU70* gene encoding one of the components of the non-homologous end joining system is required, which necessitates the use of a first selection marker. The cleavage of the target gene at each end using a dual RNA-guided Cas9 complex, followed by recombination with a repair template containing the hygromycin resistance gene, allowed disruption of the target gene in the *ΔKU70* mutant. Four genes encoding dioxygenases, catalyzing the critical ring-opening step in aromatic hydrocarbons, were successfully disrupted, and the optimum efficiency was observed using 5 µg of the *HygR* repair cassette. Alternatively, in the wild-type strain, the exclusive use of two Cas9 RNP complexes was enough to achieve an efficient deletion method; one dioxygenase gene was successfully deleted in up to 20% of the obtained colonies. These last experimental conditions path the way to multiple gene deletions and complementation experiments, which cannot be reached using our first procedure since only two selection markers are available for *Scedosporium* species.

Keywords CRISPR-Cas9 · *Scedosporium apiospermum* · Fungal genome editing · *in vitro* strategy · Optimization

Introduction

Scedosporium species are saprophytic soil filamentous fungi. Although they are widely distributed in the environment, they displayed a particular tropism for polluted and anthropized environments [1]. They can also behave as opportunistic pathogens in receptive hosts [2]. Nowadays, seventeen species are recognized in the *Scedosporium* genus [3–6], but only seven of them have been described in human infections (*i.e. S. angustum*, *S. apiospermum*, *S.*

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aurantiacum, *S. boydii*, *S. dehoogii, S. ellipsoideum*, and *S. minutisporum)* [3, 7]. They may cause a wide range of infections, from localized infections in immunocompetent hosts to severe disseminated infections in immunocompromised patients [2]. Respiratory colonization / infections are also frequently reported in patients with pre-existing disorders, like bronchiectasis or sarcoidosis. In addition, the genus *Scedosporium* ranks the second among the filamentous fungi colonizing the airways of patients with cystic fibrosis, after *Aspergillus fumigatus*. Due to their low susceptibility to all systemic antifungals available, these filamentous fungi are difficult to treat.

The rapid development of high-throughput sequencing techniques and their democratization due to lower costs have facilitated large-scale investigation of various biological processes. Currently, several high-quality *Scedosporium* genomes are publicly available, some of them being well-annotated, which enables further investigations on fungal pathogenicity [8–11]. As filamentous fungi have a genetic background more complex than bacteria, their genetic manipulation often is difficult. However, molecular manipulation of genes and proteins are essential to elucidate their function in an organism. The limited number of efficient genetic engineering tools explains the slow progress made in understanding the pathogenic mechanisms in *Scedosporium* species. Although genetic transformations have been reported, targeted gene disruption and integration of a DNA donor into the host genome remain a great challenge in this genus. The non-homologous end joining (NHEJ) is the dominant mechanism over homologous recombination; therefore, ectopic integrations are especially common [12–15].

In recent years, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated RNA-guided Cas endonuclease has become the leading tool for genome editing in a wide range of species [16]. The single-guided RNA (sgRNA), resulting from the combination of a target specific CRISPR RNA (crRNA) and a nuclease-binding trans-activating crRNA (tracrRNA), associated with the Cas9 nuclease, enables the enzyme to cleave the target sequence and to generate a double-strand DNA break (DSB). The recognition of the DNA target region depends on a protospacer adjacent motif (PAM), which is a nucleotide triplet NGG located immediately at the 3' end of the genomic target region [17, 18]. DBS repair is mediated by two major pathways: NHEJ pathway and homology directed repair (HDR) [19, 20]. NHEJ is an error-prone repair mechanism that commonly results in short insertions or deletions (INDELs) of nucleotides [21, 22], whereas the HDR pathway is an accurate repair system, involving the annealing with homologous sequences (usually > 100 bp) from a DNA template. However, two alternative pathways relying on homologous sequences of different lengths were also reported: the microhomology-mediated end joining (MMEJ) requires micro-homologous sequences (2–20 bp) while single strand annealing (SSA) uses longer homologous sequences (> 25 bp) [20, 21, 23, 24].

CRISPR-Cas9 exhibits a huge potential in fungal research and has already been successfully used in several yeasts and filamentous fungi. Various methods have been developed in filamentous fungi, using in vivo expression strategy [25, 26] as well as in vitro assembled ribonucleoprotein (RNP) complex [27, 28].

In a previous study, we demonstrated the technical feasibility of implementing in vitro CRISPR-Cas9 technology in the *Scedosporium* genus [15]. However, optimization steps are essential to make this tool reliable and accurate as well as easy-to-use and fast. Orthologs to four dioxygenases characterized in other filamentous fungi were selected in *S. apiospermum* to perform this optimization. Previously, these four dioxygenases were identified as key-enzymes within the lower funneling pathways, catalyzing the critical ring-opening step in aromatic hydrocarbons [29], which could explain the common occurrence of *Scedosporium* species in polluted environments.

Materials and Methods

Strains and Culture Conditions

The wild-type (WT) strain of *S. apiospermum* used in this study, initially isolated from a sputum sample from a patient with cystic fibrosis (CF) in 1998, was deposited at the BCCM/IHEM culture collection (Brussels, Belgium) under the accession number IHEM 14462 [8]. The non-homologous

end-joining-deficient strain (*ΔKU70)* was obtained previously from this WT strain [13].

Strains were maintained by regular passages on YPDA medium (yeast extract 10 g/L, peptone 20 g/L, agar 20 g/L) containing 0.05% chloramphenicol. For cultivation of the *ΔKU70* mutant, the culture medium was supplemented with phleomycin (50 µg/mL), whereas hygromycin B (50 µg/mL) was also added to the culture medium for cultivation of the double mutants to maintain the selection pressure.

Genomic DNA Extraction

Genomic DNA of the obtained transformants was extracted from colonies grown on YPDA (supplemented with hygromycin B at 50 µg/mL) for 7 days at 37°C. The mycelium was ground in liquid nitrogen and the crushed material was resuspended in lysis buffer (10 mM Tris–HCl; 1 mM EDTA; 2% Triton X-100; 1% SDS; 0.1 M NaCl, pH 8). DNA was extracted with phenol/chloroform before being precipitated with ethanol. The contaminating RNA were digested by incubation for 1 h at 37°C with RNase A 0.2 mg/mL. DNA quantification was performed on Qubit 2.0 Fluorometer, and integrity was checked by 1% agarose gel electrophoresis. Genomic DNA was stored in TE buffer (10 mM Tris–HCl pH 7.5; 1 mM EDTA pH 8) at 4 °C.

Design of crRNA Protospacers and in vitro Assembly of Cas9 RNP Complexes

Cas9 RNP complexes were composed of the protein Cas9 Nuclease V3, the universal tracrRNA (5'GTT TTAGAGCTAGAAATAGCAAGTTAAAATAAG GCTAGTCCGTTATCAACTTGAAAAAGTGGC ACCGAGTCGGTGCT3') and a crRNA specific to the target region. One or two crRNA per target gene were designed to direct Cas9 cleavage within the 5' end or within the 5' and 3'ends of the coding sequence, respectively. First, all PAM sites (usually NGG) located in these gene regions were identified. Then various softwares (Benchling, Chopchop, and ATUM) were used to define the optimal crRNA sequences [30, 31]. Finally, a BLAST search in the *S. apiospermum* reference genome (JOWA00000000.1 from strain IHEM 14462) was performed using each protospacer sequence to minimize off-target cleavage (Table S1).

The three RNP components were supplied by Integrated DNA Technologies (IDT). Stock solutions of crRNA and tracrRNA (100 µM) were prepared by dilution of lyophilizates in Nuclease-Free Duplex buffer and stored at—20 °C until use. For generation of the sgRNA, each crRNA was hybridized with the universal tracrRNA by mixing equimolar amounts of these compounds in nuclease-free duplex buffer to obtain a final concentration of 33 µM. The mix was boiled at 95°C for 5 min, then cooled at room temperature for 15 min and stored on ice for up to 4 h until transformation. Cas9 RNP complexes were prepared according to the method developed by Al Abdallah et al. for *A. fumigatus* [27]: 1.5 µL of sgRNA (33 µM) were added to 0.75 µg of Cas9 protein (2.32 nM) in a final volume of 10 µL. Reaction was incubated at room temperature for 10 min. An in vitro test was performed on a plasmid including the target sequence to confirm the cleavage efficiency of the RNP complexes.

Construction of the HygR Repair Cassettes

The HygR sequence (1646 bp), including a partial sequence of the *Aspergillus nidulans* gpdA promoter, the hygromycin B transferase gene *Hph*, and the *A. nidulans* trpC terminator, was obtained by PCR from the plasmid pCB1636 [12]. It confers hygromycin B resistance which was used as selection marker for disruption throughout this work.

DNA donor cassettes were composed of the HygR sequence flanked by 80 bp sequences homologous to the target gene region. Amplification was performed using Q5 high-fidelity DNA polymerase (New England Biolabs), and amplicons were purified on gel using the NucleoSpin Clean-up Kit (Macherey–Nagel) (Table S2).

Homology cassettes for disruption of *SAPIO_CDS4203* gene were composed by the HygR sequence flanked by longer homologous sequences (between 500 to 1000 bp). The NEBuilder HiFi DNA Assembly technology (New England Biolabs) based on the seamless assembly of multiple DNA fragments was used according to the manufacturer's instructions. Four DNA fragments amplified by PCR using primers with short overlapping sequences (15–30 bp) were joined (Table S2): a 5' recombination sequence (1 kb), the HygR sequence (1646 bp), a 3' recombination sequence (1 kb) and the

pKS plasmid sequence (4,133 kb). They were mixed at equimolar ratio (with a total amount of 0.5 pmols) in the Master Mix and incubated at 50°C for 60 min. A heat shock transformation of competent *E. coli* DH5α was performed using the resulting plasmid. All constructions were validated by sequencing.

Transformation

Transformation was performed as previously described, using the standard PEG-$CaCl_2$ chemical procedure [12] on 10^7 protoplasts from *S. apiospermum* IHEM 14462 wild-type strain (WT) or its derived *ΔKU70* mutant, with 1 or 5 µg of DNA donor cassette and 10 µL of the RNP complex. Putative fungal transformants were picked and sub-cultured on YPDA supplemented with hygromycin B. Further purification was performed by a round of single-spore isolation and three successive subcultures. For the WT strain, transformation was also performed in the absence of DNA donor cassette on three different quantities of protoplasts (10^4, 10^5 and 10^7); in this case, the transformants were picked and sub-cultured on YPDA without drugs. All the transformants were validated by PCR using relevant primer combinations (Table S3).

Results

Gene Deletion Favors the Integration of the DNA Donor Cassette in the *ΔKU70* Strain

To favor the integration of the DNA donor cassette by homology direct repair, experiments were first performed in the *ΔKU70* mutant, deficient in the NHEJ system. Two different strategies were developed and tested on the *SAPIO_CDS0603* gene (encoding gentisate 1,2 dioxygenase): *i*) the use of a unique in vitro assembled Cas9 RNP which allows a double-strand DNA break at the 5' end of the gene; and *ii*) the use of an in vitro assembled dual RNA-guided Cas9 (dual Cas9 RNP) complex to delete the gene and replace it by the DNA donor cassette (Fig. 1a).

Transformed protoplasts were recovered on hygromycin-containing agar plates. The simple gene disruption failed to produce hygromycin-resistant transformants, whatever the Cas9 RNP complexes used. Conversely, several hygromycin-resistant transformants were obtained using dual Cas9 RNP complexes: 74 with 0603_P1 and 0603_P4 Cas9 RNPs, and 45 with 0603_P4 and 0603_P9 Cas9 RNPs (Table 1). A PCR was performed on the hygromycin-resistant colonies to discriminate wild-type (1922-pb amplicon) *vs.* one-copy DNA donor cassette insertion (2485-pb amplicon) (Fig. 1b). All the transformants produced a PCR band consistent with a single insertion of the DNA donor cassette at the target locus (Fig. 1c), which was confirmed by sequencing analysis of the relevant locus for four of the transformants.

We also examined the effect of the amount of DNA donor cassette on the number of the obtained transformants with effective replacement of the target gene by performing transformation experiments with two different quantities of the repair cassette (*i.e.*, 1 µg and 5 µg). There was no qualitative effect on the gene replacement efficiency, which means that, as described previously, all the hygromycin-resistant transformants displayed a replacement of the *SAPIO_CDS0603* gene by the DNA donor cassette. However, compared to the use of 5 µg of DNA donor cassette, a reduced number of transformants was obtained when using only 1 µg of DNA donor cassette (Fig. 1d).

On the *ΔKU70* parent strain, we also attempted to disrupt three other genes encoding dioxygenases: *SAPIO_CDS4203* (Protocatechuate 3,4 dioxygenase), *SAPIO_CDS8719* (Catechol 1,2 dioxygenase) and *SAPIO_CDS9498* (Hydroxyquinol 1,2 dioxygenase). These three genes encode proteins exhibiting similar enzymatic activities, but they are located at different loci on the reference genome. Two dual Cas9 RNP complexes cleaving at each end of the target coding sequence were designed for each gene (Table S1). In Table 1 are indicated the numbers of the obtained transformants with replacement of the target gene by the DNA donor cassette validated by PCR. An evident gene-dependent effect was observed since we obtained 43 and 19 transformants for *SAPIO_CDS4203*, 0 and 5 for *SAPIO_CDS8719*, and 3 and 2 for *SAPIO_CDS9498*, according to the dual Cas9 RNP complexes used. As reported in other organisms, these results suggest the editing efficiency is not similar across the genome.

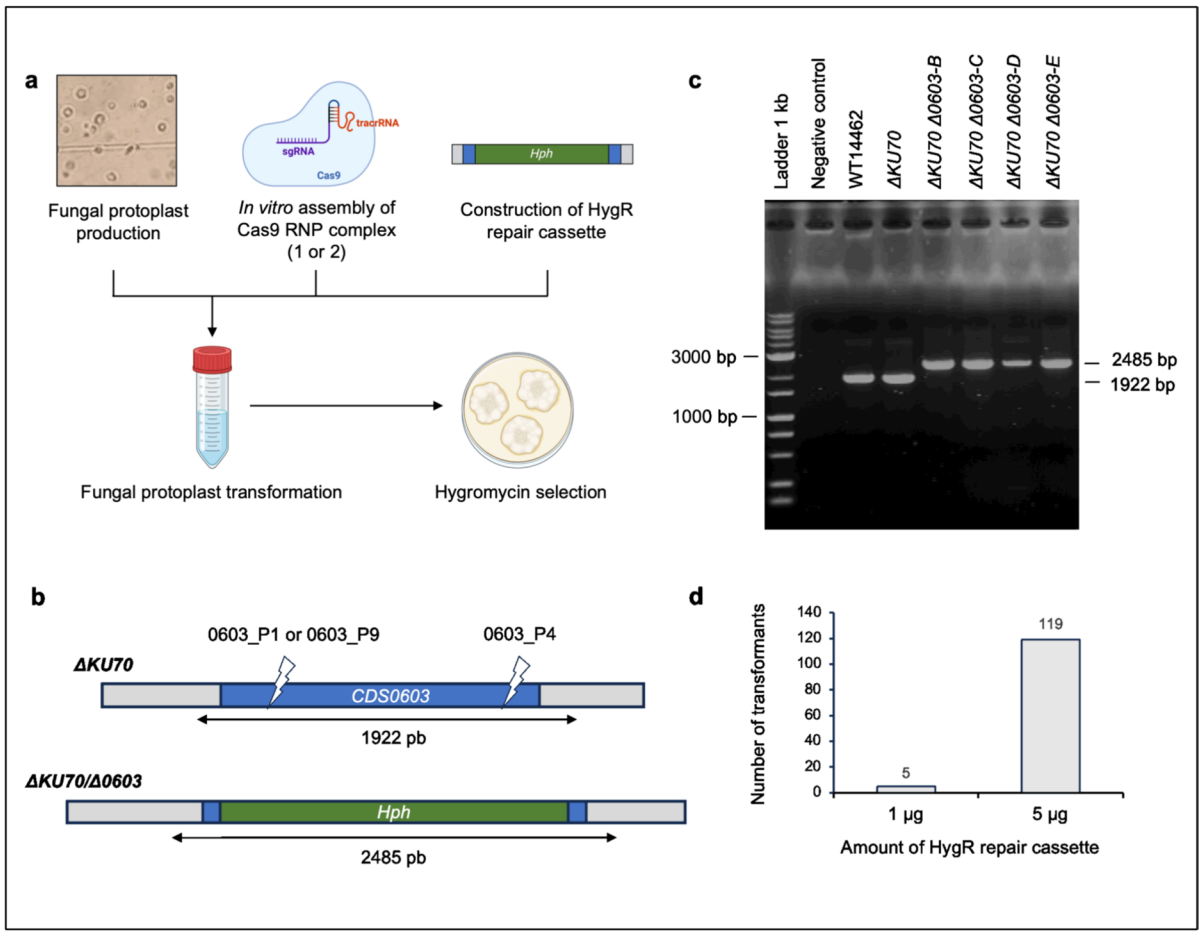

Fig. 1 PCR validation of the *ΔKU70/Δ0603* mutants obtained using dual Cas9 RNP complexes. **a** Schematic diagram of CRISPR-Cas9 RNP mediated genome editing through protoplast transformation in the *S. apiopermum ΔKU70* mutant used as parent strain. **b** Map of the *SAPIO_CDS0603* locus and strategy for the replacement by the DNA donor cassette. The DSBs created by CRISPR-Cas9 RNP complexes are positioned on the target gene and the size of the expected PCR fragment is indicated by arrows. **c** PCR analysis of the *ΔKU70/Δ0603* transformants. **d** Effect of DNA donor cassette quantity on the number of transformants with an effective replacement of the target gene. The number of transformants corresponds to the sum of transformants obtained in 3 independent experiments

Table 1 Number of transformants and total number of colony-forming units (CFU) obtained with the *ΔKU70* mutant as parent strain for the four target genes tested using two different dual Cas9 RNP complexes

The number of transformants corresponds to the sum of transformants obtained in 3 independent experiments

Targeted gene		Dual RNA complexes used	Positive CFU / total CFU
CDS number	Gene function		
SAPIO_CDS0603	Gentisate 1,2 dioxygenase	0603_P1 and 0603_P4	74 / 74
		0603_P4 and 0603_P9	45 / 45
SAPIO_CDS4203	Protocatechuate 3,4 dioxygenase	4203_P1 and 4203_P3	43 / 43
		4203_P1 and 4203_P4	19 / 19
SAPIO_CDS8719	Catechol 1,2 dioxygenase	8719_P1 and 8719_P3	0 / 0
		8719_P2 and 8719_P3	5 / 5
SAPIO_CDS9498	Hydroxyquinol 1,2 dioxygenase	9498_P1 and 9498_P3	3 / 3
		9498_P2 and 9498_P3	2 / 2

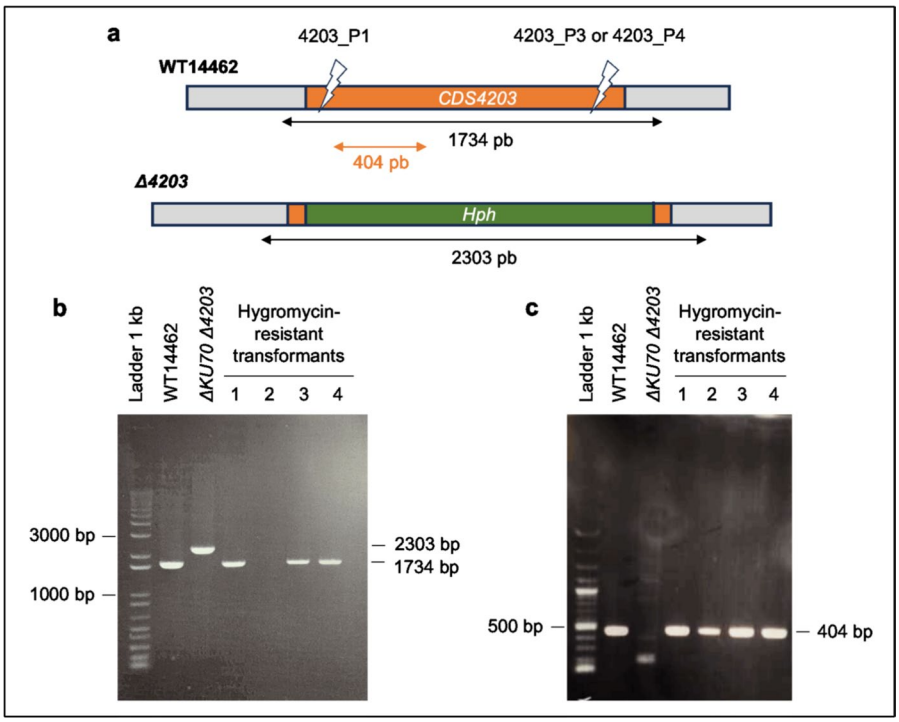

Fig. 2 PCR analysis of the transformants obtained using dual Cas9 RNP complexes from the wild-type strain IHEM 14462 with a DNA donor cassette. **a** Map of the *SAPIO_CDS4203* locus and strategy for the replacement by the DNA donor cassette. The DSBs created by CRISPR-Cas9 RNP complexes are positioned on the target gene. The black arrows define the fragments expected in PCR for validation of the deletion mutants. The orange arrows define an internal fragment of the target gene of which the detection by PCR demonstrates the absence of deletion of the target gene. **b** PCR analysis of the hygromycin-resistant transformants. **c** PCR demonstrating the absence of target gene deletion

Genome Editing May be Achieved in the Absence of a Donor DNA Cassette in *S. apiospermum* WT Strain

The CRISPR-Cas9 technology developed and optimized on the *ΔKU70* mutant as parent strain was tested on the WT strain. For this experiment, we selected the *SAPIO_CDS4203* gene (encoding the protocatechuate 3,4 dioxygenase), for which a moderate efficacy was obtained for genome edition in the *ΔKU70* mutant (Table 1). To genotype the hygromycin resistant colonies, PCR was used to discriminate between wild-type gene (1734-bp amplicon) *vs.* one-copy DNA donor insertion (2303-bp amplicon) (Fig. 2a). Among the 84 hygromycin-resistant transformants, 53 were analyzed by PCR. 55% of them (29/55) displayed a wild-type PCR band suggesting an ectopic insertion of the cassette (Fig. 2b). For the remaining transformants analyzed, PCR results indicated mainly an absence of amplification (78%) or showed a PCR band with an unexpected size (lower in most cases, but sometimes higher). Another PCR was performed using primers located inside the target gene, *i.e. SAPIO_CDS4203* gene. For all transformants, the gene was not deleted (Fig. 2c).

In an attempt to improve the positivity rate, a DNA donor cassette with longer homologous sequences (500 to 1000 bp length sequences instead of the usual 80-bp length) was constructed and tested on the WT strain. However, most of the obtained transformants also showed ectopic insertions and PCR always revealed the presence of the target gene (data not shown). Because of this, same disruption experiments were carried out without the DNA donor cassette. In these conditions, we no longer had a selection marker to identify the transformants. Therefore, the quantity of protoplasts used was reduced in order: (i) to limit the number of colonies obtained at the end of the experiment; and (ii) to be able to analyze by PCR a representative proportion of randomly selected colonies. The two dual Cas9 RNP complexes (at an

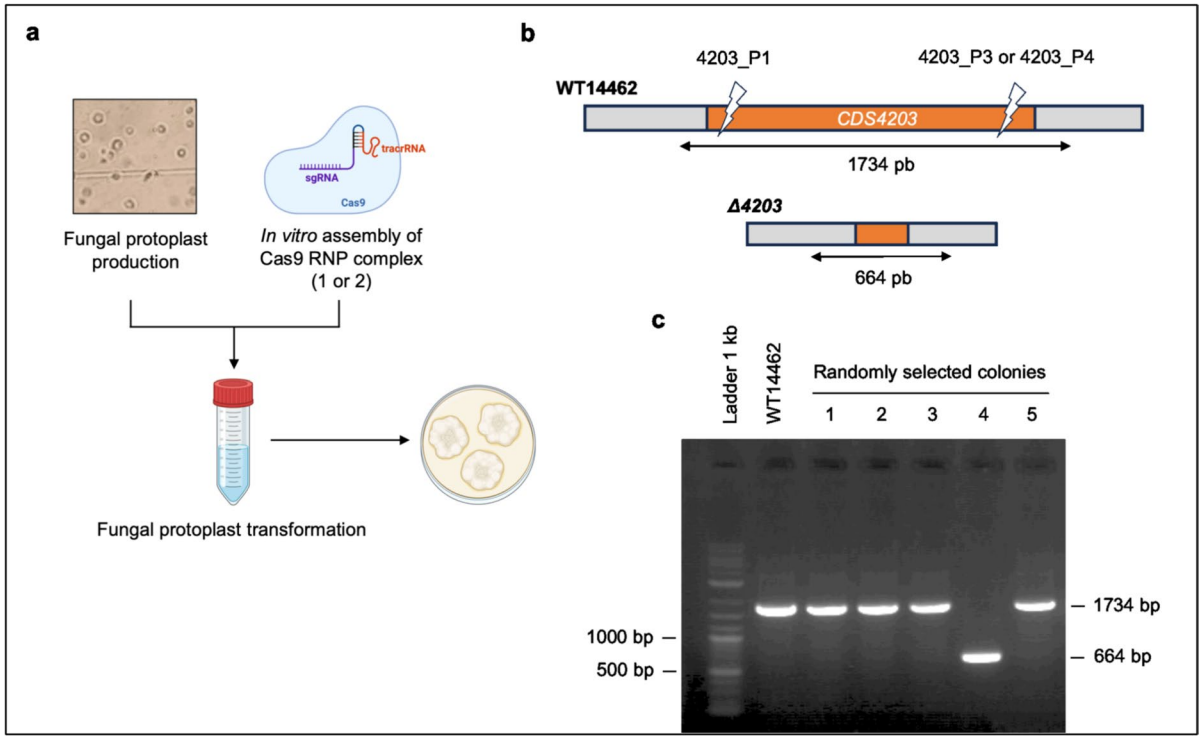

Fig. 3 PCR analysis of the transformants obtained using dual Cas9 RNP complexes from the wild-type strain IHEM 14462 in the absence of a DNA donor cassette. **a** Schematic diagram of CRISPR-Cas9 RNP mediated genome editing through protoplast transformation in the parent strain *S. apiopermum* WT14462. **b** Map of the *SAPIO_CDS4203* locus and strategy for gene deletion. The DSBs created by CRISPR-Cas9 RNP complexes are positioned on the target gene. The black arrows define the fragments expected in PCR for validation of the deletion mutants. **c** PCR analysis of five randomly selected colonies showing the successful deletion of the target gene in one of them

Table 2 Rate of transformants with deletion of the target gene according to the quantity of cells per transformation

Protoplasts (cell/mL)	Dual RNP complexes used	CFUs	Positivity rate (%)
10^4	4203_P1 and 4203_P3	74.5	20.00
10^4	4203_P1 and 4203_P4	72	21.03
10^5	4203_P1 and 4203_P3	533.5	8.91
10^5	4203_P1 and 4203_P4	551	3.45
10^7	4203_P1 and 4203_P3	1.35×10^5	0
10^7	4203_P1 and 4203_P4	1.53×10^5	0

The number of CFU corresponds to the mean number of CFUs obtained in two independent experiments

CFU: Colony-forming unit

unchanged concentration) which previously demonstrated their efficiency for disruption of the *SAPIO_CDS4203* gene (for example, the dual Cas9 RNP complex 4203_P1 and 4203_P3 which allowed the recovery on hygromycin-containing agar, of 43 CFUs that were all validated by PCR), were tested without the DNA donor cassette on three different concentrations of protoplasts (Fig. 3a).

PCR was used to discriminate between a wild-type genotype (1734-bp amplicon) *vs.* deletion of the gene (664-bp amplicon and 339-bp amplicon with the dual Cas9 RNP complexes 4203_P1 / 4203_P3 and 4203_P1 / 4203_P4, respectively) (Fig. 3b). This analysis revealed the deletion of the target gene in some colonies obtained after transformation (Table 2 and Fig. 3c), and among them, three were confirmed by sequencing analysis of the relevant locus. The ratio of dual Cas9 RNP complexes concentration to protoplast concentration influenced the positivity rate (*i.e.* deletion of the target gene). A higher ratio resulted in a higher positivity rate. Up to 20.7% of the colonies (12 mutants confirmed by PCR among the 58 colonies

analyzed) displayed deletion of the target gene when a cell density of 10^4 protoplasts per mL was used. This finding indicates that 1 to 2 validated mutants (*i.e.* with deletion of the target gene) could be obtained without addition of any exogenous DNA from the analysis of 10 randomly selected transformants.

Discussion

CRIPSR-Cas9 technology has drastically improved the efficiency of genome editing. Its molecular components, the endonuclease Cas9 and the sgRNA, are often delivered by vectors carrying expression cassettes. However, their transformation as an in vitro-assembled Cas9 RNP complex is an interesting alternative approach, widely used in animal cells [32, 33] and successfully applied in various fungal organisms [27, 28, 34, 35]. This approach provides a robust and cloning-free way, has the advantage of not requiring the integration and the continual expression of CRISPR-Cas systems, and consequently can overcome cytotoxicity due to the continual expression of Cas nucleases reported in some models [35–37]. In this study, we optimized this alternative method for *S. apiospermum,* targeting genes encoding four dioxygenases identified as key-enzymes within the lower funneling pathways in lignin degradation [29]. However, different strategies appear necessary depending on whether the NHEJ system is functional or not.

Regarding filamentous fungi, in the first report of the in vitro use of Cas9 RNP complexes, several genes were deleted from a NHEJ-deficient *P. chrysogenum* strain using 1000-bp flanking sequences in the DNA donor cassette [34]. Two years later, efficient gene replacement was obtained in *A. fumigatus* using a CRISPR-Cas9 system based on a dual-guided RNA Cas9 complex, and a DNA donor cassette flanked by 35 to 50 bp of homology [27]. As the NHEJ is the dominant mechanism over HDR in *S. apiospermum* [12], we first performed our experiments in a NHEJ-deficient strain (*ΔKU70* parental strain), using 80-bp flanking sequences in the DNA donor cassette. Unlike single-guided RNA Cas9 complexes which failed to generate transformants, dual Cas9 RNP complexes that cleave at the ends of the target gene, gave interesting results. The use of dual Cas9 RNP complexes ensures the elimination of all gene isoforms, whereas the insertion of a DNA donor cassette with a selection marker in a single site may only break the open reading frame (ORF) of certain gene isoforms [27]. The replacement of the target gene was observed in all transformants and the number of transformants was positively correlated to the amount of DNA donor cassette. However, the efficiency varied according to the target gene. In other model systems, physical and genomic features such as chromatin structure or chromosome location have been reported to affect the choice of DNA repair pathway [19, 20, 38, 39]. Histone H3 lysine 36 modification has been extensively studied in yeast for its role in DNA repair [40]. In *Schizosaccharomyces pombe*, its methylation promotes NHEJ system while its acetylation promotes homologous recombination [41]. Thus, depending on the locus and its environment, the implementation and efficiency of homologous recombination may be impacted. Moreover, the use of chemical agents such as inositol or benomyl to synchronize cells and control the cell cycle should be considered to further improve transformation efficiency in *ΔKU70* strain. The choice of repair system partly depends on the phase of the cell cycle: the NHEJ route can occur in the G1 phase whereas the HDR system occurs only during DNA replication [42]. Indeed, an increased ratio of edited homokaryotic transformants of *A. oryzae* was observed after adding reagents related to mitosis and cell cycle (from 0 to 40% for inositol and 71.43% for benomyl) [43].

Transposing this functional protocol to the wild-type strain IHEM 14462 provided opposite results. Ectopic insertions of the DNA donor cassette were frequent, due to the functional NHEJ systems. In the other transformants, unexpected results were observed (characterized by an absence of amplification by PCR or the detection of an amplicon with an unexpected size). In addition, the target gene was conserved in all hygromycin-resistant transformants. One hypothesis to explain these results is the occurrence of insertion of one or more DNA cassettes (partially or totally) at one of the DSB generated. Abnormal genotyping results with negative PCR or detection of an amplicon with a size larger than expected were reported previously in genome editing in other filamentous fungi, such as *Pyricularia oryzae* (formerly *Magnaporthe oryzae*) [44], *A. fumigatus* [26] and *Trichoderma reesei* [45]. Gene deletion without replacement overcomes this

drawback. Such experiments should be performed on a reduced number of protoplasts, the ratio between concentration of the dual Cas9 RNP complexes and the number of protoplasts being a crucial element. Complementary experiments should be conducted to assess whether the efficiency varies depending on the target gene, as in the *ΔKU70* strain. In addition, only two selection markers are available for *Scedosporium* species, phleomycin already used for disruption of the *KU70* gene in the WT strain and hygromycin, which prevents multiple gene disruptions as well as complementation after disruption of a target gene in the *ΔKU70* background; therefore, the absence of a selection marker frees us from these constraints and paths the way for the obtention of multiple deletion mutants and complementation experiments.

Author Contributions Kévin Ravenel performed the scientific monitoring, data recovery and participated in the writing of the draft. Wilfried Poirier and Bienvenue Razafimandimby performed some scientific experiments. Jean-Philippe Bouchara provided the funding and was responsible for the final version of the paper. Amandine Gastebois and Sandrine Giraud designed and supervised the study and participated in the writing of the paper.

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Declarations

Competing Interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Approval Not applicable because the study does not involved human and/or animal participants.

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