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A CRISPR Cas12a/Cpf1 strategy to facilitate robust multiplex gene editing in *Aspergillus Niger*

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Abstract

Background CRISPR technologies have revolutionized strain engineering of *Aspergillus* species, and drastically increased the ease and speed at which genomic modifications can be performed. One of the advantages of CRISPR technologies is the possibility of rapid strain engineering using multiplex experiments. This can be achieved by using a set of different guiding RNA molecules (gRNA) to target multiple loci in the same experiment. Two major challenges in such experiments are firstly, the delivery of multiple guides simultaneously, and secondly, ensuring that each target locus is cut efficiently by the CRISPR nuclease. The CRISPR nuclease Cas12a, also known as Cpf1, presents a unique advantage to bypass this challenge. Specifically, and unlike Cas9, Cpf1 is able to release several gRNAs from a common precursor RNA molecule through its own RNase activity, eliminating the need for elements such as ribozymes or tRNA machinery for gRNA maturation. This feature sets the stage for much more straightforward construction of vectors for the delivery of many gRNAs, which in turn allows each locus to be targeted by multiple gRNAs to increase the odds of successfully inducing a break in the DNA.

Results Here we present a toolbox that can be used to assemble plasmids containing a gRNA multiplex expression cassette, which is able to express a multi gRNA precursor. The precursor can be processed via Cpf1 RNase activity to produce multiple functional gRNAs in vivo. Using our setup, we have constructed plasmids that are able to deliver up to ten gRNAs. In addition, we show that three simultaneous deletions can be introduced robustly in *Aspergillus niger* by targeting each gene with several gRNAs, without prior gRNA validation or the use of genomically integrated selection markers.

Conclusion In this study we have established an efficient system for the construction of CRISPR-Cpf1 vectors that are able to deliver a large number of gRNAs for multiplex genome editing in *Aspergillus* species. Our strategy allows multiple specific genomic modifications to be performed in a time frame of less than two weeks, and we envision this will be able to speed up cell factory construction efforts significantly.

Keywords CRISPR gene editing, Cpf1, Cas12a, *Aspergillus Niger*, Multiplexing, Fungi

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Background

Aspergilli have been utilized in industrial biotechnology for over a century [1, 2]. Originally, they were used for food fermentation (well-known examples include miso, soy sauce, and saké), citric acid production, and manufacture of native enzymes such as amylases [1, 2]. Over recent decades the product repertoire has expanded to include other organic acids and heterologous proteins with commercial value [1–3]. However, construction and optimization of new cell factories not only requires integration of the genes necessary to make the product, but also numerous strain specific genetic modifications to improve product yield, stability, purity, and safety [3–5]. This process typically takes several years [6] and accordingly, there has been a constant urge to develop more efficient genetic engineering methods for fungi. This has resulted in tools such as non-homologous end-joining (NHEJ) deficient strains, allowing more efficient homologous recombination [7–10], and simplified assembly of gene-targeting substrates from PCR fragments either in vitro or in vivo [11–13]. In particular, the introduction of CRISPR technologies in fungi has dramatically increased the efficiency of gene targeting experiments, including even the introduction of marker free modifications [14, 15]. Importantly, the potential of CRISPR technology has not been fully exploited. The vast majority of fungal CRISPR experiments are using an iterative step-by-step engineering pipeline, with multiple rounds of singleplex gene-targeting. This results in long timelines for cell factory construction. CRISPR multiplexing could solve this problem, but its implementation in fungi is still in its infancy. The challenge is to simultaneously deliver multiple gRNAs to target the CRISPR nuclease to introduce DNA double-stranded breaks (DSBs) at the relevant targets within the genome. In fungi, gRNAs are typically delivered via a plasmid which expresses them from a gRNA expression cassette. Using this setup, there are several reports of successful multiplexing in *Aspergilli* by expressing 2–5 gRNAs from a single extra-chromosomal vector [16–21].

CRISPR mediated editing depends on the efficiency of the individual gRNAs used, and multiplexing is severely hampered if one or more gRNAs are not able to efficiently mediate DSBs. Even in singleplex experiments, reliable editing requires identification of efficient gRNAs, which can be achieved by individually pre-testing gRNAs in e.g. TAPE (Technique to Assess Protospacer Efficiency) experiments [16, 22]. However, this process is laborious and time consuming, limiting the time benefit gained from performing multiplex editing. As a faster alternative, it may therefore be attractive to use multiple different un-tested gRNAs to target each locus, increasing the odds that target loci are all successfully cleaved, see Fig. 1A. With this strategy, the production of many different gRNAs to target multiple genes is the challenge. Multiple gRNAs can be released from a single precursor RNA molecule if they are separated by spacers that can be removed by RNase activity. For the widely employed CRISPR nuclease Cas9 this may be accomplished by using self-cleavable ribozymes, or tRNA sequences that can be processed by the cells native tRNA processing machinery [16–18, 21, 23, 24]. However, the repetitive use of large spacers to separate gRNA coding sequences complicates construction of the gRNA multiplex expression cassette (MEC), and the cassette may even be genetically unstable due to direct repeat recombination of the homologous elements. The CRISPR nuclease Cas12a/Cpf1 (henceforth Cpf1) can potentially alleviate this problem. Unlike Cas9, Cpf1 can liberate gRNAs from pre-crRNA via its own RNase activity, specifically cleaving RNA at a small 14–16 nt stem-loop in the 19–20 nt conserved repeat of the gRNAs [25–27]. Importantly, efficient Cpf1 based CRISPR technology has already been implemented in different species of *Aspergillus* [28, 29]. Moreover, the endogenous gRNA release mechanism of Cpf1 was recently used in *Aspergillus oryzae* to liberate two different gRNAs from a common precursor RNA [19]. As a result, mutations were introduced at the two gRNA target loci, presumably due to flawed NHEJ repair. In this paper, we take this strategy to the next level and demonstrate it is possible to release sets of gRNAs from a precursor RNA to specifically modify multiple loci in *Aspergillus niger* through homologous recombination (HR) mediated gene targeting.

Results and discussion

A Cpf1 based method for multiplexing gene editing in Fungi

We have developed a system to deliver multiple gRNAs for multiplex gene editing in fungi. Our system is based on a set of plasmids, each with different fungal markers (*pyrG*, *argB*, *hph* and *ble*), which we have previously used for Cpf1 mediated fungal gene editing via a single gRNA [28]. These plasmids all contain an AMA1 sequence for fungal replication [30], a codon optimized and NLS extended version of *Lachnospiraceae bacterium cpf1* for Cpf1 production [28], and a uracil-specific excision reagent (USER)-cloning cassette to allow for simple insertion of a gRNA expression cassette by *Escherichia coli*-based USER fusion [31].

Originally, the vectors were used to deliver a single gRNA, released from a precursor RNA via tRNA processing machinery. For this, an expression cassette controlled by the *Aspergillus fumigatus* U3 promoter (U3p) and terminator (U3t) was inserted into the USER cassette of a vector. The gRNA expression cassette was constructed by fusing two USER compatible PCR fragments: one that contained the U3p, a tRNA, and the 5' section of the

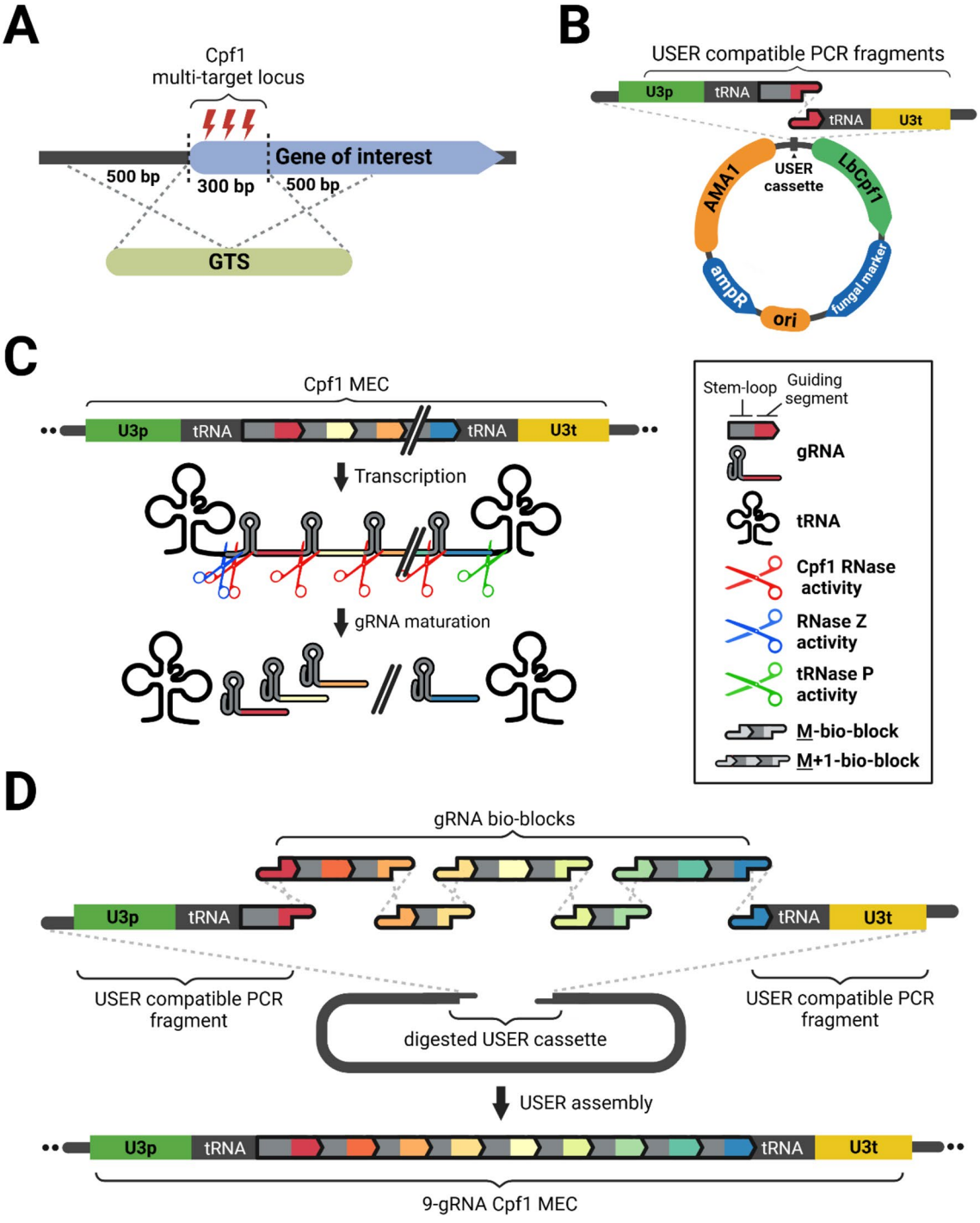

Fig. 1 *Strategy for Cpf1 multiplex CRISPR editing.* ***A****Schematic depiction of gene knock-out with a gRNA redundancy strategy. Multiple gRNAs are used to induce a double stranded break. Homology-directed repair using a dsDNA gene targeting substrate (GTS) as template induces a 300 bp deletion at the start of the gene.* ***B****Schematic for USER assembly of a Cpf1 CRISPR vector containing one gRNA using two USER compatible PCR fragments.* ***C****Schematic depiction of expression of a Cpf1 multi-guide precursor RNA from a MEC controlled by a U3 promoter and terminator, followed by processing of the precursor RNA by Cpf1, RNase Z and tRNase P to yield mature gRNAs.* ***D****Schematic USER assembly of a 9-gRNA Cpf1 MEC using two USER compatible PCR fragments and five gRNA bio-blocks.* Graphics legend is presented in the box

gRNA and another that contained the 3' section of the gRNA, a tRNA, and the U3t, see Fig. 1B. For sequences of the specific elements, see Additional file1: Table S1. When these two PCR fragments were merged by USER fusion, the guiding segment of the gRNA was assembled by the sequences in the primer tails, allowing programming of the gRNA.

In this study, we devised a strategy to expand this system to deliver multiple gRNAs for Cpf1 multiplexing. For this, gRNA arrays were designed expressing nine to ten guide RNAs as a single precursor RNA molecule, flanked by tRNA sequences. After transcription of the precursor RNA, the flanking tRNA sequences are removed through the RNase Z and tRNase P activities of the cell, while Cpf1 cleaves upstream of the gRNA stem-loops to release mature gRNAs, see Fig. 1C.

Like in the original system, the promoter, the two tRNAs and the terminator are delivered via two PCR fragments. To assemble multiple guides into this cassette, the USER compatible PCR fragments now contain overhangs complementary to the first or last gRNA in the array, respectively. A series of dsDNA oligonucleotides (referred to in this study as bio-blocks) with relevant complementary single-stranded overhangs are then used to assemble the array of gRNA sequences into the cassette. To ensure USER assembly occurs in a defined order, the overhang sequences reside in the unique guiding segment of the gRNAs. In this way, sequential fusions of all the gRNA bio-blocks and the USER compatible PCR fragments lead to the assembly of the complete gRNA MEC, see Fig. 1D. The content of a bio-block may vary. In their minimal form, they are composed of a single stem-loop sequence flanked by parts of variable gRNA segments (*M*-bio-blocks). However, one or more additional full-length gRNA sequences can be added to increase gRNA content per bio-block. In the experiments presented, we used CRISPR vectors made from *M*-bio-blocks, or bio-blocks containing a single additional full-length gRNA sequence (*M* + 1-bio-blocks). In most cases, we used an assembly strategy where the two types of gRNA bio-blocks, five in total, were fused in an alternating manner to create a MEC containing nine gRNAs, see Fig. 1D.

Construction of multi-gRNA expression cassettes

To explore the efficiency of this method, we performed three multiplex experiments, where the aim of each experiment was to simultaneously delete ~ 300 bp, including the start codon, of three different genes in *A. niger* using a single CRISPR vector. In our experiments, we employed three gRNAs for each gene, so each CRISPR vector expresses a precursor RNA containing nine gRNAs. Moreover, each gRNA was designed to target Cpf1 to a sequence within the ~ 300-bps region intended for deletion. Specifically, we set out to mutate three different sets of genes, for simplicity named set 1 (gene A, B, and C), set 2 (gene D, E, and F), and set 3 (G, H, and I). Selected genes were judged to be unlikely to produce a sick or deleterious mutant phenotype. Actual gene names, predicted functions and the gRNA target sequences, including *P*rotospacer *A*djacent *M*otifs (Cpf1 requires TTTY as *PAM*), are provided in Additional file 2: Table S2. CRISPR vectors used to target gene set 1 (pCpf1.MEC1) and 3 (pCpf1.MEC3) were constructed using the pAC1430 (*pyrG*) CRISPR backbone, whereas the vector used for targeting set 2 (pCpf1.MEC2) was based on the backbone of pAC1749 (*hph*). In all three cases, the vectors were made in a single step by the orderly fusion of a linearized vector backbone, two USER compatible PCR fragments, and five gRNA bio-blocks, as shown in Fig. 1D.

To explore the upper limit of the system, we also constructed a vector that was designed to induce DNA DSB formation in five more genes (set 4: Gene J, K, L, M and N, see Additional file 2: Table S2) by expressing only two gRNAs per target gene. Assembly of the CRISPR vector containing the ten-gRNA MEC needed for this experiment (pCpf1.MEC4) was achieved in a one-step USER fusion scheme, where four *M* + 1-bio-blocks and one *M*-bio-block were fused and inserted into the USER cassette of pAC1430 (*pyrG*) to complete the MEC, see Additional file 3: Figure S1. For all four CRISPR vectors we were able to identify correct constructs by screening five to ten *E. coli* transformants with diagnostic PCR, followed by Sanger sequencing.

The successful one step assembly of up to ten gRNAs into a single array demonstrates the effectiveness of the described USER cloning approach for the construction of multi-gene targeting CRISPR-Cpf1 plasmids.

Multiplex gene deletions in *A. niger*

To achieve accurate deletions after Cpf1 cleavage, we employed dsDNA gene targeting substrates (GTSs) consisting of the fused 500 bp flanks up- and down-stream of the planned deletion, Fig. 1A. We performed co-transformations of *A. niger* NIG159 protoplasts with each of the three CRISPR plasmids, targeting gene-sets 1–3 respectively (pCpf1.MEC1, pCpf1.MEC2 and pCpf1.MEC3), along with the appropriate GTSs in triplicate. As a control, each CRISPR plasmid transformation was also performed without GTSs. As NIG159 is NHEJ deficient, DSBs in the absence of a repair template will be lethal, since the cell has no ability to repair the break without a homologous GTS [16]. Thus, this serves as a control to validate that these plasmids can introduce one or more DSBs. As expected, these controls produced no growth (Fig. 2A) indicating that at least one functional gRNA was released from each of the precursor gRNAs.

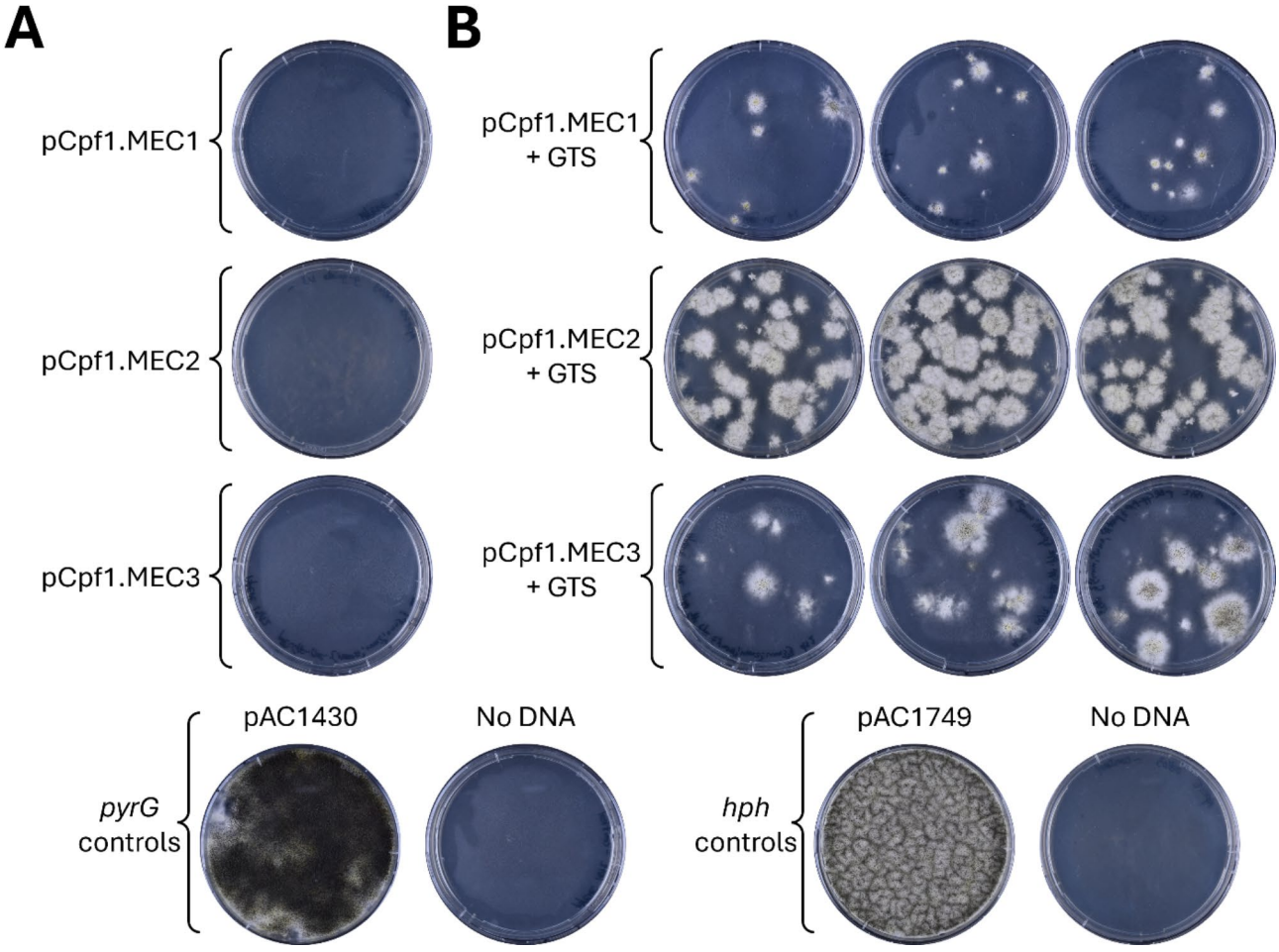

Fig. 2 *Three-target Cpf1 multiplex gene deletions in* ***A. niger***. ***A****Transformations with three-target Cp1 multiplex vectors (pCpf1.MEC1, pCpf1.MEC2 or pCpf1.MEC3), without GTS molecules.* ***B****Co-transformations with three-target Cpf1 multiplex vectors and appropriate GTS molecules*

After appropriate selection was applied, the transformations produced between 6 and 33 healthy-looking colonies for each gene set (Fig. 2B). For each experiment several transformants were then randomly selected and genotyped with diagnostic PCR (Additional file 4: Figure S2 and Additional file 5: Table S3).

For the co-transformations using pCpf1.MEC1, diagnostic PCRs were examined for the ~300 bp deletion within genes A, B, and C (gene set 1). This showed 17 out of 21 screened transformants were likely homokaryons and contain all three deletions. Three additional transformants also contained all three deletions, but appeared heterokaryotic as the PCR also produced a band corresponding to wild-type genotype for all three genes. The last transformant had the expected deletions in gene A and B but harbored a larger than expected, approximately 800 bp, deletion in gene C.

Screening of 21 transformants from the co-transformation experiments using pCpf1.MEC2 (gene set 2) showed that all 21 strains appeared homokaryotic as PCRs yielded one band. Eight out of the 21 transformants contained all three deletions. The remaining 13 had only two deletions; they showed deletion bands for genes D and E, but the wild-type band for gene F.

For the co-transformation experiments using pCpf1.MEC3 (gene set 3), we analyzed 18 transformants by diagnostic PCR. For gene G this demonstrated that all 18 transformants contained the deletion. However, for gene H and I the outcomes of the PCR reactions were more complicated. Firstly, for gene H, 13 transformants produced an unexpected band pattern, and for gene I, 8 transformants produced no bands. Since the analyses for gene G were all successful, these results are likely not due to DNA quality. PCRs for 9 and 10 transformants showed deletion and/or wild-type alleles of gene H and I respectively. We therefore hypothesize that the unexpected results represent aberrant repair of Cpf1 induced DSBs e.g. by other NHEJ independent mechanisms such as microhomology mediated end-joining (MMEJ) [32]. Despite this, we identified six transformants containing all three deletions, and three of these contained no additional aberrant or wild-type amplicons. The full

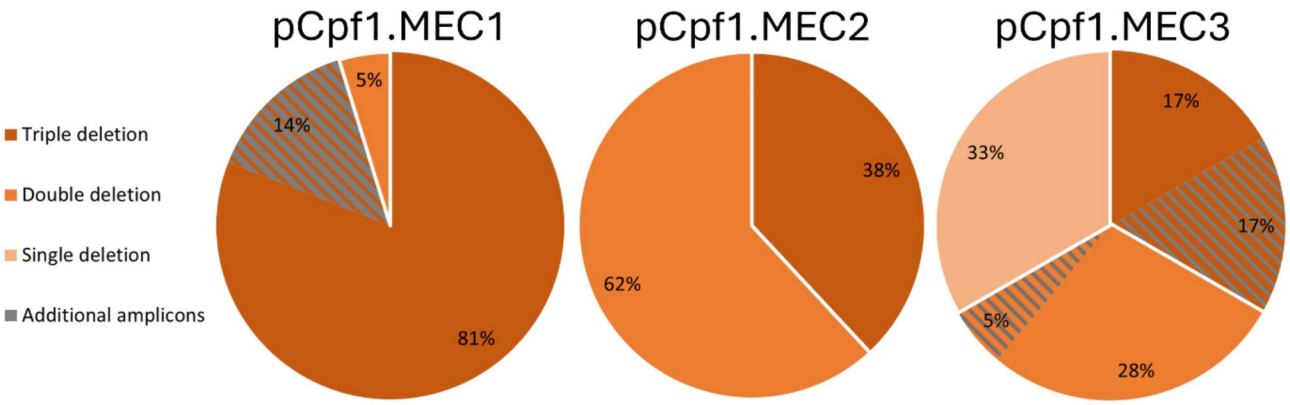

Fig. 3 Rate of genotype frequencies found in triple deletion transformations based on diagnostic PCR. 'Additional amplicons' represents a wild-type or aberrant amplicon in addition to the deletion amplicon. No transformants without deletions were observed. (pCpf1.MEC1 n = 21, pCpf1.MEC2 n = 21, pCpf1.MEC3 n = 18)

distribution of events observed in the 3-target experiments can be seen in Fig. 3.

Altogether, these experiments demonstrate it is possible to robustly introduce three specific genomic modifications using CRISPR-Cpf1 induced HR by using three unvalidated gRNAs for each target gene. The efficiency of producing likely homokaryotic strains containing all three deletions in sets 1, 2, or 3, were 81%, 38%, and 17% respectively (Fig. 3).

The efficiency of the co-transformation experiment with the CRISPR plasmid expressing the ten-gRNA MEC, pCpf1.MEC4 (gene set 4) was low. Only six healthy transformants were obtained in the three independent trials. However, like the experiments with the other plasmids, the absence of GTSs further reduced viability to one transformant, indicating the release of at least one functional gRNA (Additional file 6: Figure S3A). All six transformants were examined for the presence of the intended deletions by diagnostic PCR. (Additional file 6: Figure S3B and Additional file 5: Table S3). This indicated all six were homokaryons, of which three contained three deletions, two contained two deletions, and one contained one deletion. The set of transformants did not contain the combination of all deletions, rather covering four genes total. Therefore, we assume it is possible to produce a quadruple deletion mutation with this setup. For all the transformants, one or more of the diagnostic PCR reactions failed. For example, an amplicon for gene L was not obtained in any of the transformants. Like with gene-set 3, we hypothesize this may be caused by aberrant DNA repair. To this end, we envision that the chance of forming undesired repair products may increase with the number of DSBs and target genes, perhaps due to increased stress on the cell, leading to increased use of alternative repair pathways like MMEJ [32]. Moreover, the low number of transformants indicates a high level of lethality after transformation, likely reflecting that one of the Cpf1 induced DNA DSBs escaped repair. Altogether, our experiments indicate that up to four genes can be deleted simultaneously using this Cpf1 multiplexing system but aiming for the modification of more than three genes via multiple uncharacterized gRNAs may require time-consuming additional transformations and screening and will reduce the robustness of the pipeline.

Conclusion and perspectives

Here we present an AMA1 vector-based strategy for efficient Cpf1 multiplex CRISPR editing in *A. niger*, which utilizes a combination of native Cpf1 RNase activity and tRNA processing to release gRNAs from a single precursor RNA. We show this strategy can be used to simultaneously perform marker-free targeted deletions in at least three loci, without relying on lengthy prior validation of functional gRNAs. This accelerates complex strain construction work compared to methods relying on pre-validated guides, only allowing single edits, or requiring marker recycling for each round of transformation. Strain engineering speed could be increased even further by employing in vivo DNA assembly for vector construction to completely omit bacterial cloning [11]. Construction of MECs with many gRNAs may prove a barrier in large scale experiments where modified strains need to be made in parallel. Using larger bio-blocks with more than one complete gRNA could mitigate this challenge. To this end we note that the size of these larger bio-blocks can potentially be reduced, since we have used 23-nt targeting sequences and shorter 19-21-nt sequences have been applied successfully in mammalian cells [33, 34]. Furthermore, novel DNA synthesis technologies, for example terminal deoxynucleotidyl transferase mediated synthesis, may help with the assembly of complex arrays by making synthesis of larger gRNA bio-blocks available in the future [35, 36]. Additionally, previous studies show that gRNA attenuation can improve

genome editing efficiency in bacteria [37]. If the number of simultaneously targeted loci is limited by a low rate of HR based repair, resulting in low transformant viability, then gRNA attenuation could improve transformation efficiency by lowering the rate at which DSBs are induced by Cpf1. This would reduce the load exerted on the HR machinery of the cell by CRISPR-Cpf1 induced DSBs. HR capacity could also be expanded, for example by recovering transformants in the presence of HR inducing molecules [38, 39], via enhancement of HR machinery [40], or by incorporation of a more efficient heterologous system [41]. Finally, in case there is an upper limit to the number of DSBs which can be efficiently repaired in a single experiment, DSB independent CRISPR editing via catalytically altered Cpf1 base editors could be pursued [42, 43]. Although this will not allow for large deletions, point mutations can be introduced to inactivate a gene. We note that this strategy may also be useful if the task is to change the function of multiple proteins simultaneously. Altogether, we envision that the methodologies presented in this report will be able to speed up cell factory construction significantly.

Materials and methods

Strains and media

E. coli strain DH5α was used for cloning and to propagate plasmids, in liquid or on solid (2% agar, Sigma-Aldrich) Luria broth medium supplemented with 100 µg/mL ampicillin (Sigma-Aldrich). *A. niger* strain NIG159 (*pyrG1*, *kusAΔ*, IS1::A-P*gpdA-uidA*-T*trpC*-B [44]) was used for all fungal experiments, and was cultivated in liquid or on solid (2% agar) glucose based minimal medium [45] supplemented with10 mM uridine (uri) (Sigma-Aldrich) and 10 mM uracil (ura) (Sigma-Aldrich). NIG159 transformants were recovered on sucrose-based transformation medium (TM) [45] without supplements for experiments with *pyrG* as selection marker or on TM supplemented with 10 mM uri, 10 mM ura and 80–400 µg/mL hygromycin B (hygB) (Roche) for experiments with *hph* as selection marker.

PCR

Primers were manufactured by Integrated DNA Technologies (IDT), listed in Additional file 7: Table S4. PCR reactions were performed at 50 µL volume. (Reaction composition: 20 mM Tris-HCl pH 8.0 (ThermoFisher), 10 mM KCl (ThermoFisher), 6 mM $(NH_4)_2SO_4$ (Sigma-Aldrich), 2 mM $MgSO_4$ (Sigma-Aldrich), 50 µg Bovine Serum Albumin (Sigma-Aldrich), 0.1% Triton x-100 (Sigma-Aldrich), 0.5 µM forward and reverse primer, 0.2 µM dNTPs (ThermoFisher), 1 U *PfuX7* polymerase [46], 10–100 ng template DNA). Standard PCR reactions used a 35-cycle touchdown program [47], with annealing temperature starting at 68°C and decreasing by 0.5°C each cycle. PCR cloning fragments were purified with a NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). Fragments for vector construction were obtained using plasmid DNA templates. PCR for GTS construction or screening of fungal transformants used fungal genomic DNA as template. For *E. coli* colony PCR, a single colony was touched with a pipette tip and resuspended in 20 µL MilliQ water (MQ). 1 µL of this suspension was used as template.

Plasmid construction

Plasmids constructed and used in this study are listed in Additional file 8: Table S5. All *Cpf1* CRISPR multiplexing vectors were constructed by USER cloning [31] using vector backbones of pAC1430 and pAC1749 [28], linearized by double digestion with *PacI* and *Nt.BbvCI* (New England Biolabs, NEB) according to manufacturer's specifications. PCR fragments for vector assembly were generated using a previously constructed CRISPR plasmid containing a single gRNA (pAC1441 [28]) as template and USER cloning compatible *PfuX7* polymerase [46] and were treated with USER enzyme (NEB). After assembly, plasmids were screened by *E. coli* colony PCR and correct assembly was verified through Sanger sequencing (Mix2Seq, Eurofins Genomics).

gRNA bio-block annealing

Oligonucleotides used for bio-block assembly were manufactured by IDT and are listed in Additional file 9: Table S6. gRNA Bio-blocks with ssDNA-ends were constructed by incubating a 50 µL reaction mix containing two largely complementary single-stranded oligonucleotides (0.3 µM of each oligonucleotide, 1x CutSmart buffer (NEB), MQ) in a thermal cycler, starting at 95°C for 4 min before ramping down from 70°C to 25°C over 2 h. 3 µL of the resulting reaction mix was used for USER assembly.

Extraction fungal DNA for PCR

Fungal DNA was obtained by using a pipette tip to scrape mycelium from a 3-day old colony and lysing the material with lysis buffer (1 mM EDTA, 100 mM NaCl (Sigma-Aldrich), 10 mM TRIS-HCl, 1% SDS (Sigma-Aldrich), 2% Triton x-100) and 200 µL 0.1–0.25 µm glass beads (Retch) in a FastPrep Cell Disrupter (Savant Bio 101 FP120) at speed 4.0 for 40 s. 15 µL 5 M NaCl and 400 µL ice-cold 96% ethanol (Sigma-Aldrich) was added to 150 µL of lysate supernatant to precipitate the DNA, which was then air dried for 15 min and resuspended in 150 µL MQ.

Gene targeting substrate construction

For each target gene, two ~ 500 bp PCR fragments were amplified from the up- and downstream regions of the intended deletion with primers that contained tails to

introduce a ± 24 bp overlap between the fragments. The overlapping region was then fused in a two-step overlap extension PCR [48, 49] starting with 15 cycles at 55ºC annealing temperature without primers, followed by 30 cycles of standard touchdown PCR with primers.

Fungal transformation

NIG159 protoplasts were generated according to the method described by Nielsen et al. [12]. For *pyrG* based transformations, 10^6-10^7 protoplasts, 1 µg Cpf1 multiplex vector and 0.8 pmol of each GTS was mixed with 150 µL PCT and incubated for 10 min at room temperature before adding 250 µL ATB and transferring to TM plates with appropriate selection. Transformations with *hph* as selection marker were performed according to the two-phase protocol described by Vanegas et al. [50] with TM bottom and top layer supplemented with 80 µg/mL and 400 µg/mL hygB respectively. For all experiments, transformations with CRISPR vector and GTSs were performed in triplicate. Additionally, a transformation with a Cpf1 vector carrying the selection marker and no gRNA expression cassette was included as control for protoplast recovery and a transformation without DNA was included as control for the selection marker. Diagnostic PCR was performed with primers that bind outside of the regions overlapping with the GTS to verify successful targeted gene editing in the transformants.

Abbreviations

DSB	DNA double-stranded break
GTS	gene targeting substrate
gRNA	guide RNA
HR	homologous recombination
hygB	hygromycin B
IDT	Integrated DNA Technologies
MEC	gRNA multiplex expression cassette
MMEJ	microhomology mediated end-joining
MQ	MilliQ water
NHEJ	non-homologous end-joining
NEB	New England Biolabs
TM	transformation medium
uri	uridine
ura	uracil
USER	uracil specific-excision reagent
U3p	Aspergillus fumigatus U3 promoter
U3t	Aspergillus fumigatus U3 terminator

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40694-025-00196-7.

Additional file 1: Table S1. A word file (Additional file 1.docx) that contains Table S1: Cpf1 multiplex expression cassette features

Additional file 2: Table S2. A word file (Additional file 2.docx) that contains Table S2: Gene names, predicted functions and protospacer sequences

Additional file 3: Figure S1. A word file (Additional file 3.docx) that contains Figure S1: Schematic for the USER assembly of a 10-gRNA Cpf1 multiplex vector

Additional file 4: Figure S2. A word file (Additional file 4.docx) that contains Figure S2: Genotyping of transformants from triple deletion experiments with diagnostic PCR

Additional file 5: Table S3. A word file (Additional file 5.docx) that contains Table S3: Deletion experiment genotype frequencies based on diagnostic PCR

Additional file 6: Figure S3. A word file (Additional file 6.docx) that contains Figure S3: Five-target Cpf1 multiplex gene deletion in A. niger

Additional file 7: Table S4. A word file (Additional file 7.docx) that contains Table S4: List of primers

Additional file 8: Table S5. A word file (Additional file 8.docx) that contains Table S5: List of plasmids.

Additional file 9: Table S6. A word file (Additional file 9.docx) that contains Table S6: List of oligonucleotides for bio-block assembly

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Not applicable.

Author contributions

SMMP, FJC, BG, KER and UHM were instrumental in the conceptualization of this project. APvE, SMMP, FJC and UHM contributed to the experimental design. APvE performed all experiments. APvE, SMMP and UHM wrote this paper. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. SMMP, BG, and KER are or were employees of Better Dairy Ltd. during this work.

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