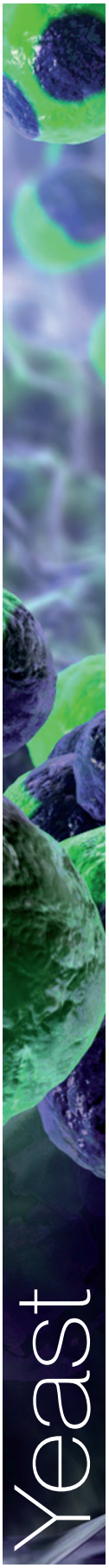

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Research Article

CRISPR/Cpf1 enables fast and simple genome editing of *Saccharomyces cerevisiae*

René Verwaal*, Nathalie Buiting-Wiessenhaan, Sacha Dalhuijsen and Johannes A. Roubos
DSM Biotechnology Center, Alexander Fleminglaan 1, 2600 MA, Delft, the Netherlands

**Correspondence to: René Verwaal, DSM Biotechnology Center, Alexander Fleminglaan 1, 2600 MA, Delft, the Netherlands. E-mail: rene.verwaal@dsm.com*

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Abstract

Cpf1 represents a novel single RNA-guided CRISPR/Cas endonuclease system suitable for genome editing with distinct features compared with Cas9. We demonstrate the functionality of three Cpf1 orthologues – *Acidaminococcus spp.* BV3L6 (AsCpf1), *Lachnospiraceae bacterium* ND2006 (LbCpf1) and *Francisella novicida* U112 (FnCpf1) – for genome editing of *Saccharomyces cerevisiae*. These Cpf1-based systems enable fast and reliable introduction of donor DNA on the genome using a two-plasmid-based editing approach together with linear donor DNA. LbCpf1 and FnCpf1 displayed editing efficiencies comparable with the CRISPR/Cas9 system, whereas AsCpf1 editing efficiency was lower. Further characterization showed that AsCpf1 and LbCpf1 displayed a preference for their cognate crRNA, while FnCpf1-mediated editing with similar efficiencies was observed using non-cognate crRNAs of AsCpf1 and LbCpf1. In addition, multiplex genome editing using a single LbCpf1 crRNA array is shown to be functional in yeast. This work demonstrates that Cpf1 broadens the genome editing toolbox available for *Saccharomyces cerevisiae*.

Keywords: **CRISPR/Cpf1; CRISPR/Cas9; single Cpf1 crRNA array; genome editing; *Saccharomyces cerevisiae***

Introduction

CRISPR genome editing systems using RNA-guided endonucleases have revolutionized genome editing since the first publications demonstrating functionality *in vitro* (Jinek *et al*., 2012) and in human cells (Cong *et al*., 2013; Mali *et al*., 2013). In particular, the class 2/type II CRISPR/Cas9 system has been rapidly characterized and developed into a range of valuable genome editing, regulation and visualization tools in a wide variety of organisms (Hsu *et al*., 2014), including the yeast *Saccharomyces cerevisiae* (DiCarlo *et al*., 2013). Using a single-guide RNA (sgRNA) composed of chimeric crRNA and tracrRNA sequence, Cas9 is directed to a target site on genomic DNA and makes a double-stranded break. In order to survive, the cell must repair this break via non-homologous end-joining and/or homologous recombination. With non-homologous end-joining, errors can be introduced, typically leading to small insertions and deletions. For homologous recombination, the cell can recombine DNA using (donor) DNA templates provided to the cell (Hsu *et al*., 2014; Sander and Joung, 2014). Target site recognition requires a protospacer adjacent motif (PAM) at the 3′ end of its target site. The widely used Cas9 protein from *Streptococcus pyogenes* (SpCas9) recognizes NGG as a PAM sequence (Sander and Joung, 2014).

Cpf1 is a new class 2/type V RNA-guided endonuclease recently discovered in several bacterial genomes and one archaeal genome (Makarova *et al*., 2015). Cpf1 was recently reclassified as

Cas12a (Makarova *et al*., 2017). CRISPR/Cpf1 genome editing has been evaluated in human cells (Zetsche *et al*., 2015; Kim D *et al*., 2016), mice (Hur *et al*., 2016; Kim Y *et al*., 2016), *Drosophila* (Port and Bullock, 2016), rice (Xu *et al*., 2017) and plant cells (Kim H *et al*., 2017; Mahfouz, 2017). Interestingly, several features of the CRISPR/Cpf1 system are different compared with CRISPR/Cas9 (Zetsche *et al*., 2015):

(1) Cpf1 recognizes T-rich PAM sequences, i.e. 5′-TTTN-3′ (AsCpf1, LbCpf1) and 5′-TTN-3′ (FnCpf1), whereas this is NGG for SpCas9. Recently, the PAM preference for AsCpf1 and LbCpf1 was proposed to be TTTV (Kim HK *et al*., 2017).
(2) Cpf1 is characterized by a PAM sequence located at the 5′ end of the target DNA sequence, where it is at the 3′ end for Cas9.
(3) Cpf1 cleaves DNA distal to its PAM after the +18/+23 position of the protospacer creating a staggered DNA overhang, whereas Cas9 cleaves close to its PAM after the -3 position of the protospacer at both strands and creates blunt ends.
(4) Cpf1 is guided by a single crRNA and does not require a tracrRNA, resulting in a shorter gRNA sequence than the sgRNA used by Cas9.
(5) Cpf1 displays an additional ribonuclease activity that functions in crRNA processing (Fonfara *et al*., 2016). This might simplify multiplex genome editing, as demonstrated by Zetsche *et al*., (2017) who used a single crRNA array to simultaneously edit up to four genes in mammalian cells. Recently, a single crRNA array was also used for multiplex genome editing of rice (Wang *et al*., 2017).

Here we explore three Cpf1 proteins evaluated by Zetsche *et al*., (2015) for genome editing of *S. cerevisiae*. We compare their editing efficiencies with the editing efficiency SpCas9 through the introduction of a yellow fluorescent protein and the introduction of a three-gene heterologous carotenoid pathway into a single genomic locus. In addition, we demonstrate that multiplex genome editing using a single LbCpf1 crRNA array is functional in yeast. This is shown by the simultaneous introduction of the three genes of the heterologous carotenoid pathway to three different genomic loci. This work demonstrates that Cpf1 is an excellent addition to the genome editing toolbox for *S. cerevisiae*, allowing the use of an alternative PAM sequence compared with the one recognized by CRISPR/Cas9. It also provides an alternative system for multiplex genome editing as compared with previously published Cas9-based multiplex approaches for yeast (Stovicek *et al*., 2017).

Materials and methods

Yeast strain

All experiments were performed in *S. cerevisiae* strain CEN.PK113-7D [*MAT*a *URA3 HIS3 LEU2 TRP1 MAL2–8 SUC2* (Van Dijken *et al*., 2000)].

Cas9 plasmid construction

The Cas9 expression cassette consists of a Cas9 variant with a C-terminal linker protein sequence and a SV40 nuclear localization signal (Mali *et al*., 2013) expressed from the Kl11 promoter (*K. lactis* promoter of KLLA0F20031g) and the *S. cerevisiae GND2* terminator. The Cas9 sequence originates from *S. pyogenes* (SpCas9). The Cas9 expression cassette was ordered at DNA2.0 (Newark, USA). The Cas9 nucleotide sequence was codon-pair optimized for expression in *S. cerevisiae* according to the method described by Roubos and van Peij (2008). The Cas9 expression cassette was KpnI/NotI ligated into pRS414 (Sikorski and Hieter, 1989), resulting in an intermediate plasmid. Subsequently, a KanMX resistance marker gene expressed from the *Ashbya gossypii* TEF promoter and terminator was NotI ligated into the intermediate plasmid, resulting in low-copy (CEN/ARS) plasmid pCSN061 (Fig. 1).

Cpf1 plasmid construction

A linear PCR fragment of the pCSN061 plasmid omitting the Cas9 expression cassette was obtained by PCR using plasmid pCSN061 as template, primers pRV073 and pRV074 (Table S1) and Phusion DNA polymerase (New England Biolabs, USA) in the reaction. *S. cerevisiae* codon-pair optimized sequences (Roubos and van Peij, 2008) encoding the Cpf1 orthologues AsCpf1, LbCpf1 and FnCpf1, including a C-terminal linker protein

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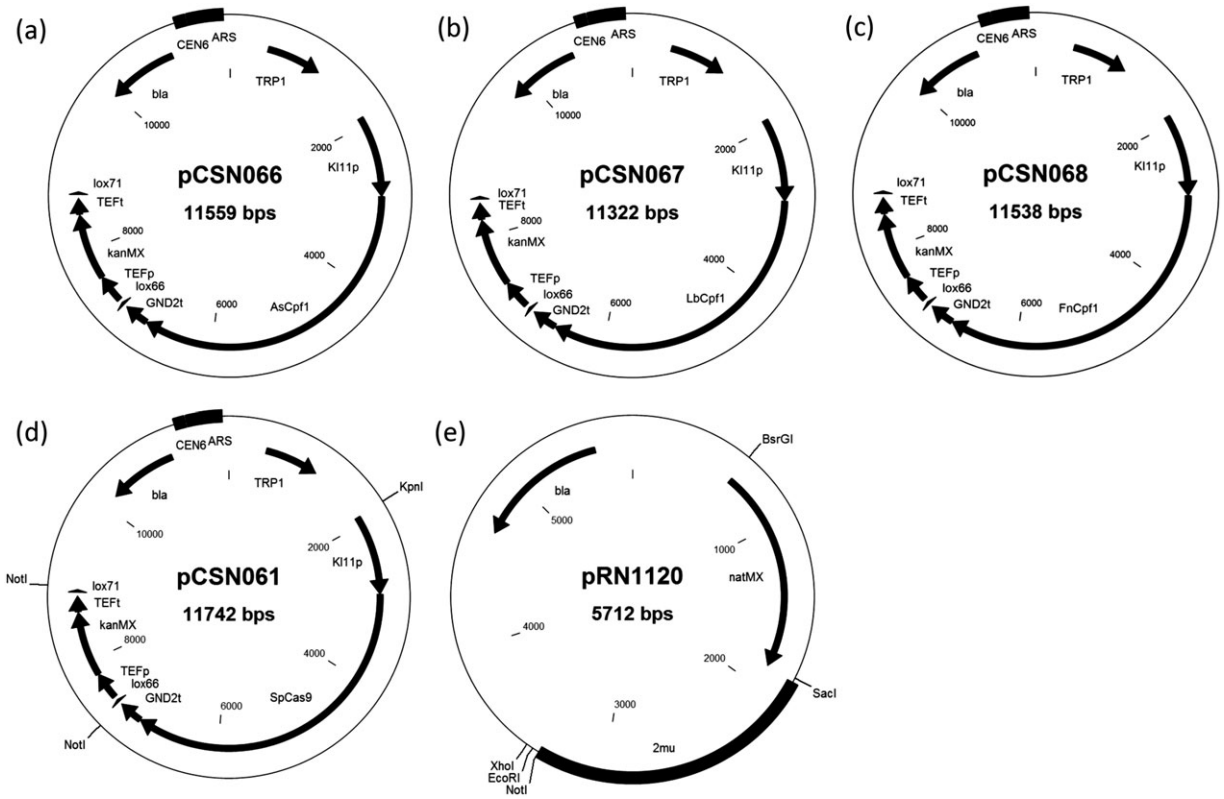

Figure 1. Maps of new plasmids for CRISPR/Cpf1 genome editing in *Saccharomyces cerevisiae*. AsCpf1 (a), LbCpf1 (b), FnCpf1 (c) and SpCas9 (d) are expressed from a low copy plasmid that includes a KanMX and *TRP1* marker. The Cpf1 or SpCas9 expression cassettes contain the *Kluyveromyces lactis* promoter (Kl11p, promoter of KLLA0F20031g) and the *S. cerevisiae GND2* terminator. Multi-copy plasmid pRN1120 (e), that includes a NatMX marker, is used for *in vivo* recombination of sgRNA or crRNA expression cassettes after linearization using EcoRI and XhoI. Bla: *E. coli* beta-lactamase gene (conferring ampicillin resistance).

sequence and SV40 NLS (Mali *et al*., 2013), were obtained as synthetic DNA at Thermo Fisher Scientific. The sequences were used as template using Phusion DNA polymerase (New England Biolabs, USA) in a PCR reaction using primers pRV081 and pRV082 for AsCpf1, pRV083 and pRV084 for LbCpf1, and pRV085 and pRV086 for FnCpf1 (Table S1, Supporting Information). Purified Cpf1 PCR fragments were individually assembled into the linear PCR fragment of the pCSN061 plasmid using Gibson assembly (Gibson *et al*., 2009). The resulting low-copy yeast expression plasmids were named pCSN066 (AsCpf1), pCSN067 (LbCpf1) and pCSN068 (FnCpf1, see Fig. 1).

Plasmid pRN1120, recipient for crRNA or sgRNA expression cassettes by *in vivo* recombination, is a multi-copy plasmid (2 μm) that contains a functional NatMX marker cassette conferring resistance against nourseothricin (Fig. 1). Plasmid pRS305 (Sikorski and Hieter, 1989) was restricted with BsrGI and NotI, removing the *LEU2* part, which was ligated with a 2 μm ORI (NotI/NgoMIV fragment) and a KanMX resistance marker gene expressed from the *A. gossypii* TEF promoter and terminator (BspEI/BsrGI fragment). The resulting plasmid was restricted with BsrGI and SacI, to remove the KanMX expression cassette, and a NatMX (nourseothricin acetyltransferase) resistance marker gene expressed from the *A. gossypii* TEF promoter and terminator (BsrGI/SacI fragment) was ligated, resulting in plasmid pRN1120 (Fig. 1).

Plasmid cloning was performed in *E. coli* NEB 10-beta competent cells (High Efficiency, New England Biolabs, distributed by Bioké, Leiden, the Netherlands). All DNA fragments were purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel, distributed by Bioké, Leiden,

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the Netherlands) according to manufacturer's instructions.

Donor DNA, crRNA, single crRNA array, sgRNA

Donor DNA expression cassettes (*crtE*, *crtYB*, *crtI*, *YFP*) codon-pair optimized for expression in *S. cerevisiae* (Roubos and van Peij, 2008) were synthesized at DNA2.0 (Newark, USA). The *crtE* expression cassette contains a *Kluyveromyces lactis TDH2* promoter and a *S. cerevisiae TDH3* terminator sequence. The *crtYB* expression cassette contains a *K. lactis YDR2* promoter and a *S. cerevisiae PDC1* terminator sequence. The *crtI* expression cassette contains a *S. cerevisiae PRE3* promoter and a *S. cerevisiae TAL1* terminator sequence. The *YFP* expression cassette contains a *S. cerevisiae TDH3* promoter and a *S. cerevisiae ENO1* terminator sequence. Donor DNA flank sequences for integration of donor DNA into genomic DNA were obtained as gBlocks (IDT, Leuven, Belgium) and then serve as templates in subsequent PCR reactions. All donor DNA expression cassette and donor DNA flank nucleotide sequences are provided in Table S2. The donor DNA expression cassettes and donor DNA flank sequences contain 50 bp connector sequences to allow for *in vivo* recombination and integration into genomic DNA as one stretch of DNA, as depicted in Figs 2 and 5. The nucleotide sequences of the connector sequences can be found in Table S2. The crRNA and sgRNA expression cassettes containing homology to the linearized pRN1120 plasmid were obtained as gBlocks [IDT, Leuven, Belgium. For nucleotide sequence information see Fig. S1 (Supporting Information) and Table S3]. The LbCpf1_crRNA_array expression cassette containing homology to the linearized pRN1120 was obtained as a gBlock (IDT, Leuven, Belgium. For nucleotide sequence information see Figs S6 and S7). Primers pRV117 and pRV118 were used to obtain a PCR fragment of a Cpf1 crRNA expression cassette, a Cpf1 single

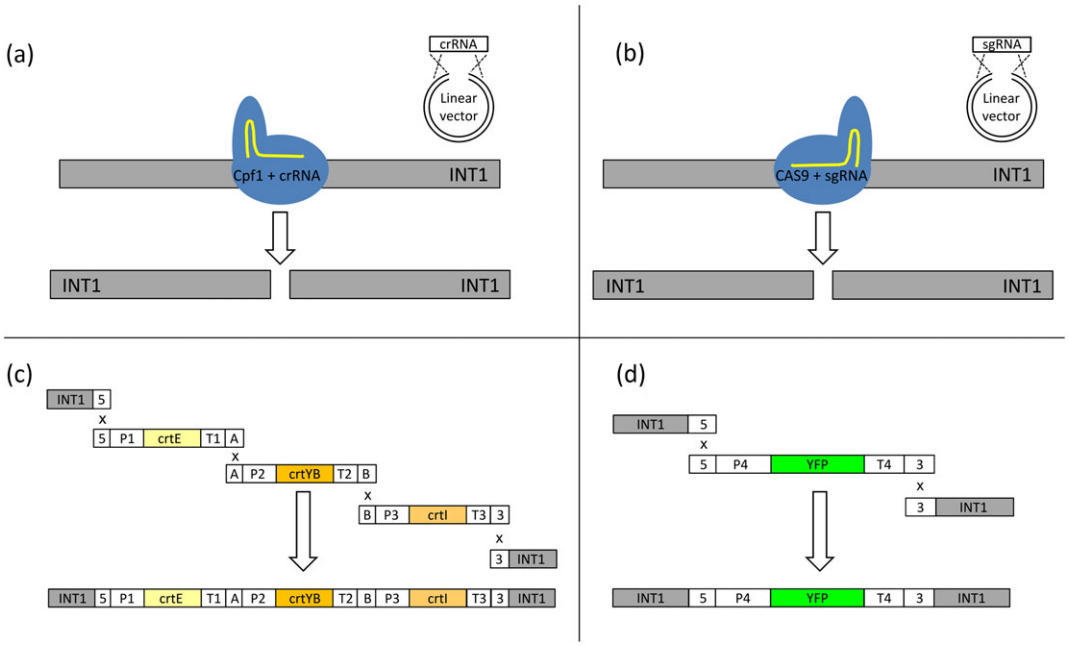

Figure 2. (a) Cpf1- or (b) Cas9-mediated genome editing approach for *S. cerevisiae*: transformation of linearized pRN1120 and a crRNA or sgRNA expression cassette containing homology with pRN1120 to allow *in vivo* recombination into a circular plasmid, to cells pre-expressing AsCpf1, LbCpf1, FnCpf1 or SpCas9. Cpf1 or Cas9 is directed to the intended genomic target site to create a double strand break. In the transformation mixture, donor DNA consisting of flank sequences and expression cassettes, carotenoid genes (c) or *YFP* (d), were included. All donor DNA assembles into one stretch of DNA into genomic DNA around the INT1 locus by *in vivo* recombination due to the presence of 50 bp homologous connector sequences, indicated as 5, A, B, C or 3. P1–P4, different promoters; T1–T4, different terminators.

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crRNA array, or a guide RNA (SpCas9) expression cassette using the gBlock as template in the PCR reaction. Using appropriate primers (Table S1) and Phusion DNA polymerase (New England Biolabs, USA) according to the manufacturer's instructions, all PCR fragments were obtained for transformation to yeast.

Yeast transformations

One microgram of the plasmids pCSN061, pCSN066, pCSN067 and pCSN068 were transformed individually to CEN.PK113-7D cells by the LiAc/SS carrier DNA/PEG method (Gietz and Woods, 2002). Each transformation mixture was plated on YPD-agar (10 g/L yeast extract, 20 g/L of peptone, 20 g/L of dextrose, 20 g/L of agar) containing 200 µg G418 (Geneticin, Sigma Aldrich, Zwijndrecht, the Netherlands) per mL. Colonies appeared on the transformation plate after 2 to 4 days' incubation at 30°C. Thereafter, from each plate, a yeast colony conferring resistance to G418 on the plate was picked, each containing an AsCpf1, LbCpf1, FnCpf1 or a SpCas9 expression plasmid, and was selected and inoculated on YPD-G418 (200 µg/mL) medium to obtain strain cultures that express AsCpf1, LbCpf1, FnCpf1 or SpCas9. Subsequently, the Cpf1 or Cas9 pre-expressing strains were transformed with (a) 100 ng of linearized pRN1120 plasmid (prior to transformation, pRN1120 was restricted with EcoRI and XhoI); (b) 750 ng PCR fragment of a crRNA (for Cpf1 orthologues), a sgRNA (for Cas9) or a LbCpf1_crRNA_array cassette (only LbCpf1 pre-expressing strains) with homology at the 5′ and 3′ end with linearized pRN1120; (c) two (singleplex) or six (multiplex) donor DNA flank PCR fragments (100 ng each) with homology to the INT1 or INT2 or INT3 integration site (singleplex), or INT1 and INT2 and INT3 integration sites (multiplex), and containing a 50 bp connector sequence to the 3′ end (left flank) or to the 5′ end (right flank); and (d) donor DNA expression cassette PCR fragments (200 ng each), being the three donor DNA expression cassette PCR fragments (encoding *crtE*, *crtYB*, *crtI*) or a *YFP* expression cassette PCR fragment (only for the singleplex experiment). The donor DNA expression cassettes contain 50 bp connector sequences to allow for *in vivo* recombination with each other and with the donor DNA flanks sequences, as depicted in Fig. 2 (singleplex approach) and Fig. 5 (multiplex single crRNA array approach).

The transformation mixtures were plated on YPD-agar supplemented with 200 µg/mL nourseothricin (NatMX, Jena Bioscience, Germany) and 200 µg/mL G418 and the plates were incubated for 2 to 4 days at 30°C.

Genomic integration (INT) loci

INT1 is a non-coding region between *NTR1* (*YOR071c*) and *GYP1* (*YOR070c*) located on chromosome XV. INT2 is a non-coding region between *SRP40* (*YKR092C*) and *PTR2* (*YKR093W*) located on chromosome XI. INT3 is a Ty4 long terminal repeat (YPRCtau3), located on chromosome XVI, and has been described by Flagfeldt *et al*., (2009).

Determination of genome editing efficiencies

The coloured colonies producing carotenoids were determined and the number was divided by the total number of transformants on a transformation plate. When *YFP* was used as donor, the numbers of fluorescent colonies were determined and divided by the total number of transformants on a transformation plate.

Correct integration of the carotenoid gene expression cassettes in the multiplex genome editing experiment, which involved the single crRNA array was determined by PCR. Genomic DNA was isolated from individual transformants according to the lithium acetate SDS method (Lõoke *et al*., 2011). Using appropriate primers (Table S1, Fig. S9) and Phusion DNA polymerase (New England Biolabs, USA) according to manufacturer's instructions, PCR reactions were performed and the PCR products were analysed on an agarose gel. When all expected PCR products were found (expected sizes are indicated in the legend of Fig. S9), the transformant was counted as correct.

Results and discussion

CRISPR/Cpf1 singleplex genome editing

We evaluated Cpf1-mediated genome editing in *S. cerevisiae* using orthologues from *Acidaminococcus* spp. BV3L6 (AsCpf1), from

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Lachnospiraceae bacterium ND2006 (LbCpf1) and from *Francisella novicida* U112 (FnCpf1) (Zetsche *et al*., 2015), by:

(1) The introduction of a heterologous metabolic pathway of three genes involved in carotenoid biosynthesis (*crtE*, *crtYB* and *crtI* from *Xanthophyllomyces dendrorhous*) (Verwaal *et al*., 2007) resulting in coloured colonies. A similar approach demonstrated functionality of CRISPR/Cas9 in *S. cerevisiae* (Jakočiūnas *et al*., 2015).
(2) The insertion of the yellow fluorescent protein (*YFP*) variant Venus (Nagai *et al*., 2002) which results in fluorescent colonies upon successful genomic insertion.

We first transformed a low-copy (CEN/ARS) KanMX-marker containing plasmid expressing codon-pair optimized AsCpf1, LbCpf1, FnCpf1 or *S. pyogenes* Cas9 (SpCas9) to the yeast cells. In a subsequent transformation, cells pre-expressing AsCpf1, LbCpf1, FnCpf1 or SpCas9 were transformed with the following components (see Fig. 2):

(1) A crRNA expression cassette for Cpf1, or a sgRNA expression cassette for SpCas9 containing homology with recipient plasmid pRN1120.
(2) The linearized multi-copy (2 μm) yeast expression plasmid pRN1120, containing a NatMX marker.
(3) Multiple donor DNA fragments for the carotenoid pathway or *YFP*, both including DNA with homology flanks to each other and/or to the genomic insertion site. For the carotenoid pathway, five donor DNA fragments were transformed. For *YFP*, three donor DNA fragments were transformed.

The crRNA expression cassette consists of the *S. cerevisiae SNR52* RNA pol III promoter (*SNR52*p), a direct repeat sequence specific for AsCpf1, LbCpf1 or FnCpf1 (Table S1), respectively, and a 20 bp spacer sequence comprising the guide-sequence (genomic target sequence, Table S3), followed by the *S. cerevisiae SUP4* terminator (*SUP4*t). *SNR52*p and *SUP4*t sequences derived from DiCarlo *et al*., (2013) were applied for both the Cpf1 crRNA and the Cas9 sgRNA expression cassettes. Examples of the nucleotide sequence of a crRNA (for Cpf1) or sgRNA (for SpCas9) expression cassette are provided in Fig. S1. crRNAs for the Cpf1 orthologues were designed for three target positions within the INT1 locus (Fig. S2). The chosen PAM sequences were in line with the Cpf1 TTTV consensus sequence (Kim HK *et al*., 2017). The three Cpf1 targeting positions were selected in close proximity to a Cas9 genomic target site (Fig. S2). Upon transformation, the linear crRNA or sgRNA cassette that contains homology with plasmid pRN1120 will assemble with the linearized pRN1120 plasmid by *in vivo* recombination (Orr Weaver *et al*., 1983). This results in a circular replicating expression plasmid allowing for selection on nourseothricin. The Cpf1- or Cas9-containing plasmids were maintained by additional selection on G418. Donor DNA consisted of two flank sequences and carotenoid gene-expression cassettes (*crtE*, *crtYB* or *crtI*), or a *YFP* expression cassette (Fig. 2). The donor DNA expression cassettes and DNA flank sequences assemble through 50 bp homologous connector sequences and integrate at the targeted INT1 locus. The INT1 flank DNA sequences were designed such that a stretch of about 1 kbp of genomic DNA is deleted upon integration of the donor DNA.

In all transformation experiments using LbCpf1, AsCpf1 and FnCpf1 orthologues, coloured or fluorescent colonies were obtained demonstrating functionality of Cpf1 in *S. cerevisiae* (Fig. 3). Three crRNA targets within the INT1 locus were evaluated for each Cpf1 orthologue. The percentage of coloured and fluorescent transformants represents the efficiency of successfully edited cells. These results demonstrate that LbCpf1 and FnCpf1 are highly functional in *S. cerevisiae*, with genome editing efficiencies comparable with SpCas9, while AsCpf1 appeared less efficient with the applied two-plasmid system. The crRNAs that target LbCpf1 and FnCpf1 to positions 2 and 3 in the INT1 locus (Fig. S2) were more effective than those that target position 1. As a negative control, AsCpf1, LbCpf1 and FnCpf1 were clearly not functional in combination with Cas9 sgRNA, and Cas9 was not functional in combination with FnCpf1 crRNA. The very low percentage of coloured or fluorescent colonies obtained is likely the result of non-CRISPR-mediated integration of donor DNA expression cassettes. Editing efficiencies for the *YFP* cassette compared with the

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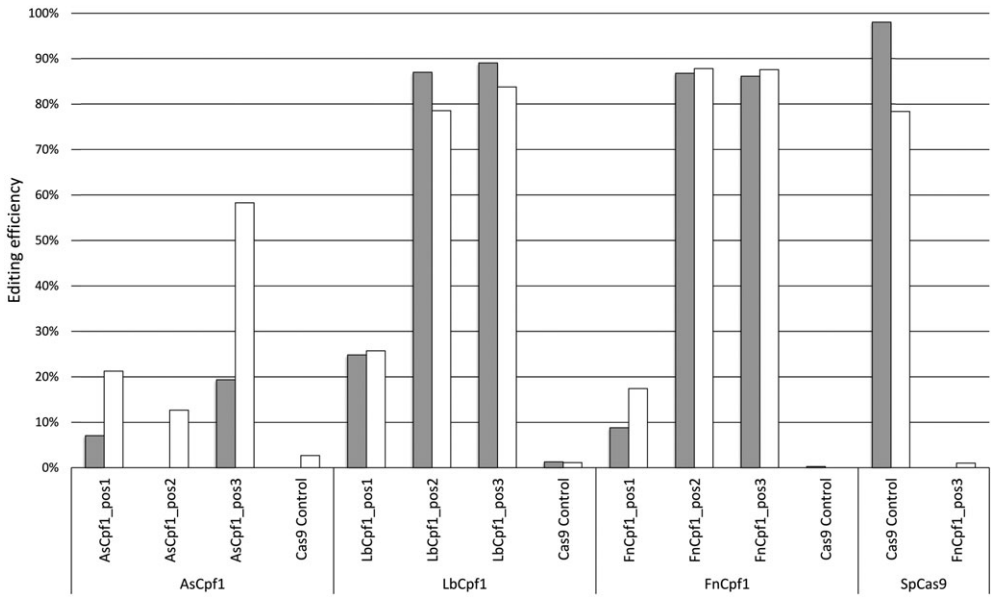

Figure 3. Percentage of successfully engineered transformants when testing different genomic target positions (n = 1), indicated as editing efficiency. Successfully engineered transformants are defined as the number of coloured or fluorescent colonies divided by the total number of transformants on a transformation plate. Grey bars: coloured transformants using carotenoid genes expression cassettes as donor DNA. White bars: fluorescent transformants using *YFP* gene expression cassette as donor DNA.

carotenoid genes cassettes were higher for AsCpf1 (13–58 vs. 0–19%) and slightly higher for FnCpf1 (17–88 vs. 9–87%), which might be due to the lower number of flank DNA and donor DNA fragments (3 vs. 5) that need to be assembled into one stretch of DNA *in vivo*.

The total number of FnCpf1 transformants was lower compared with the number of AsCpf1 and LbCpf1 transformants. In addition, the colony sizes of FnCpf1 transformants were smaller than those of the AsCpf1 and LbCpf1 (Fig. S3) edited strains, suggesting that the current FnCpf1 expression level affects the growth rate in *S. cerevisiae*. It has been reported that Cas9 expression from a high- plasmid using a strong constitutive promoter could lead to a negative influence on the growth in yeast strains (Ryan *et al*., 2014; Generoso *et al*., 2016) and we had a similar experience ourselves (unpublished results). In our experiments, the growth of the transformants was not affected by the AsCpf1 or LbCpf1 expression level, whereas FnCpf1-expressing transformants clearly showed reduced growth in comparison with the colony sizes of the AsCpf1 and LbCpf1 transformants (Fig. S3). If required, using a lower strength constitutive promoter for expression of FnCpf1 could alleviate the effect observed on the transformants. When the FnCpf1 transformation mixture was plated on a selective medium containing only nourseothricin (selecting for the crRNA plasmid), thereby relieving selection pressure for the FnCpf1 plasmid, colonies returned to the size of AsCpf1 or LbCpf1 transformants (data not shown).

Cpf1 orthologues in combination with non-cognate crRNAs

Functionality of Cpf1 orthologues in combination with non-cognate crRNAs was evaluated using the crRNAs for position 3 (Fig. S2). This position had been most functional when using cognate crRNAs for AsCpf1, LbCpf1 and FnCpf1 in combination with the carotenoid pathway at the INT1 locus (i.e, AsCpf1_INT1_pos3, LbCpf1_INT1_pos3, and FnCpf1_INT1_pos3). Again, LbCpf1 and FnCpf1 showed a higher editing efficiency compared with AsCpf1 when combined with their cognate crRNA (Figs 4 and S4). Note that the experiments in Figs 3 and 4 were performed on different days, resulting in slightly different editing efficiencies for the respective controls. Remarkably, the FnCpf1 editing efficiency

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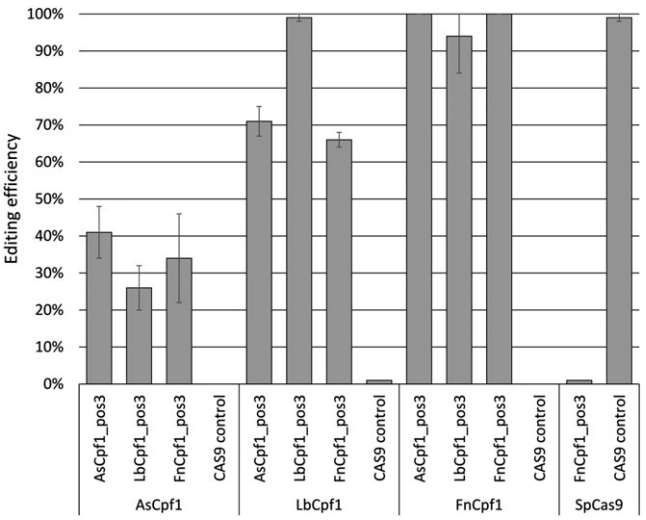

Figure 4. Percentage of successfully engineered transformants when testing Cpf1 variants with their cognate or non-cognate crRNAs (average and standard deviation depicted, $n = 3$), using carotenoid genes expression cassettes as donor DNA. Successfully engineered transformants are defined as the number of coloured colonies divided by the total number of transformants on a transformation plate.

remained similar when a non-cognate crRNA (AsCpf1_INT1_pos3 or LbCpf1_INT1_pos3) was used, whereas the genome editing efficiency of LbCpf1 clearly decreases in combination with non-cognate crRNAs (AsCpf1_INT1_pos3 or FnCpf1_INT1_pos3). Like LbCpf1, AsCpf1 displays preference for its cognate crRNA. For FnCpf1, such a clear preference was not observed, which is different from previously reported results from mammalian cell editing research, where each Cpf1 orthologue tested was most efficient with its cognate crRNA (Kim D *et al*., 2016).

CRISPR/Cpf1 multiplex genome editing using a single crRNA array

The three-gene carotenoid pathway enables the evaluation of multiplex genome editing by simultaneous integration of the individual *crtE*, *crtYB* and *crtI* expression cassettes into three individual genomic integration sites. In addition to INT1, new genomic target sites were identified for the two other genomic loci, INT2 and INT3 (Fig. S5a), by using LbCpf1 as the nuclease and by singleplex introduction and *in vivo* recombination of the *crtE*, *crtYB* and *crtI* expression cassettes into one genomic locus. The chosen PAM sequences were in line with the Cpf1 TTTV consensus sequence (Kim HK *et al*., 2017). The INT2 and INT3 flank DNA sequences were designed such that a stretch of about 1 kbp of genomic DNA is removed upon integration of the donor DNA. For the INT2 locus, all three crRNAs tested gave 100% genome editing efficiencies; for the INT3 locus, positions 1 and 2 were functional with near 100% efficiency, whereas position 3 was not functional (Fig. S5b). Subsequently, these learnings were used for the design of a single LbCpf1 crRNA array.

A LbCp1_crRNA_array expression cassette was designed as depicted in Fig. 5a. It consists of the *S. cerevisiae SNR52* RNA pol III promoter (*SNR52*p), three units of crRNAs in their mature form, a 20 bp direct repeat specific for LbCpf1 (Lb_DR) with a 23 bp guide or spacer sequence, followed by the *S. cerevisiae SUP4* terminator (*SUP4*t). *SNR52*p and *SUP4*t sequences were derived from DiCarlo *et al*., (2013). The LbCp1_crRNA_array expression cassette contains homology with plasmid pRN1120 for *in vivo* recombination. Nucleotide sequence level details of the LbCpf1_crRNA_array are provided in Fig. S6. Since research in mammalian cells has shown that shortening the direct repeat by one nucleotide (from 20 bp direct repeat for AsCpf1 to 19 bp) and using a 23 bp guide gave the best results (Zetsche *et al*., 2017), our design was adapted accordingly (Fig. S7). Using a

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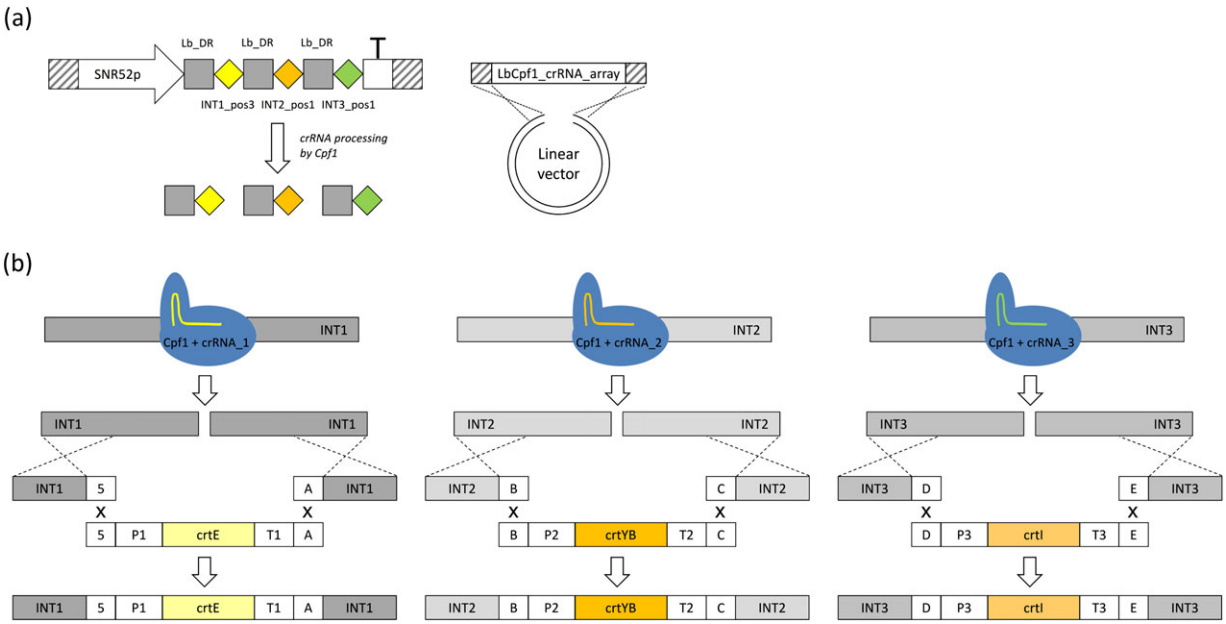

Figure 5. Schematic representation of CRISPR/Cpf1 multiplex genome editing using a single crRNA array. (a) The LbCpf1_crRNA_array is composed of three units of crRNAs in their mature form, a 20 bp direct repeat specific for LbCpf1 (Lb_DR) with a 23 bp guide sequence. Expression of the crRNA array is enabled by the *SNR52* promoter and *SUP4* terminator (T). Transformation of a linearized pRN1120 and the LbCpf1_crRNA_array that contains homology with pRN1120 (diagonal stripes) allows *in vivo* recombination into a circular plasmid, to cells pre-expressing LbCpf1. The LbCpf1_crRNA_array is subsequently processed by LbCpf1. (b) Cpf1 is directed to the intended INT1, INT2 and INT3 genomic target sites to create double stranded breaks. In the transformation mixture, donor DNA consisting of flank sequences and carotenoid gene-expression cassette were included. All donor DNA assembles into one stretch of DNA into genomic DNA around the INT1 (*crtE*), INT2 (*crtYB*) and INT3 (*crtI*) loci by *in vivo* recombination due to the presence of 50 bp homologous connector sequences, indicated as 5, A, B, C, D or E. P1–P3, different promoters; T1–T3, different terminators.

similar experimental approach as described above (paragraph 'CRISPR/Cpf1 singleplex genome editing' and depicted in Fig. 5b), flank DNA sequences and donor DNA consisting of carotenoid gene expression cassettes were transformed. Using this approach, the *crtE* expression cassette was targeted to INT1, the *crtYB* expression cassette to INT2 and the *crtI* expression cassette to INT3. A multiplex genome editing efficiency of 91 ± 5% (n = 4) was obtained using this single Cpf1 crRNA array approach. A picture of the transformants obtained is depicted in Fig. S8. No coloured transformants were obtained in control experiments where no crRNA was included or where in addition donor DNA flank sequences were left out, excluding the carotenoid gene expression cassettes from integrating into genomic DNA in a non-Cpf1 array-mediated fashion (Fig. S8). Correct integration of each of the carotenoid gene expression cassettes was verified by PCR, demonstrating that 10 out of 12 transformants had the correct integration profile (for details see Fig. S9).

Conclusions

We demonstrated that three Cpf1 orthologues (AsCpf1, LbCpf1 and FnCpf1) can be efficiently used for singleplex genome editing of *S. cerevisiae* by employing a fast and simple two-plasmid-based approach using dominant marker cassettes. In addition, our work has shown that, applying the same two plasmid-based approach, LbCpf1 is capable of multiplex genome engineering using a single crRNA array. Introduction of a crRNA expression cassette or a single crRNA array expression cassette by *in vivo* recombination into a recipient plasmid, as previously demonstrated for sgRNA for

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Cas9 (Horwitz *et al*., 2015), can also be successfully applied for CRISPR/Cpf1. This study has demonstrated functionality of the three Cpf1 orthologues and their cognate crRNAs by inserting exogenous DNA into the genome using an expression cassette (*YFP*), and in addition for the genomic insertion of a functional carotenoid pathway using a 5-fragment *in vivo* assembly approach. Throughout this work, comparisons of the CRISRP/Cpf1 system with the CRISPR/Cas9 system were made. When combined with a single crRNA array, LbCpf1 allows for efficient multiplex genome editing, shown here by the simultaneous introduction of three carotenoid gene expression cassettes into three different genomic loci.

Through the possibility to target T-rich PAM sequences, our study increases the number of potential target sites for CRISPR-mediated genome editing in yeast. In addition, CRISPR/Cpf1 allows the use of shorter guide sequences coded by a crRNA instead of the sgRNA used with Cas9. Moreover, a single Cpf1 crRNA array approach was demonstrated with high efficiency. In theory, this allows for an easier means of multiplex genome editing as compared with the previously published approaches for Cas9 (for review see Stovicek *et al*., 2017), as just one RNA pol III promoter (*SNR52*p) and one *SUP4* terminator sequence is needed for expression of a single crRNA array, which is then subsequently processed by Cpf1 (Fonfara *et al*., 2016; Zetsche *et al*., 2017). Taken together, our findings expand the genome editing toolbox available for *Saccharomyces cerevisiae*.

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Additional information

All plasmid nucleotide sequences are submitted to GenBank [pCSN061 (KY985373), pCSN066 (KY985374), pCSN067 (KY985375), pCSN068 (KY985376), pRN1120 (KY985377)]. Plasmids pCSN061, pCSN066, pCSN067, pCSN068 and pRN1120 are deposited at Addgene (www.addgene.org).

Conflict of interest

The authors declare that there is a conflict of interest. The authors have filed IP related to presented methods.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Examples of the nucleotide sequence of a crRNA (for Cpf1) or sgRNA (for SpCas9) expression cassette.
Figure S2. Graphical depiction of the INT1 integration site.
Figure S3. Pictures of transformants to determine the functionality of AsCpf1, LbCpf1 or FnCpf1 at three different genomic target positions at the INT1 locus.
Figure S4. Pictures of transformants of Cpf1 orthologs in combination with non-cognate crRNAs.
Figure S5. Graphical depiction of the INT2 and INT3 integration sites to evaluate additional genomic integration sites of other loci than INT1.
Figure S6. Nucleotide sequence of the single LbCpf1_crRNA_array expression cassette to enable multiplex genome editing in *S. cerevisiae*.
Figure S7. Comparison of the direct repeat and guide sequences present the single LbCpf1 crRNAs and in the single LbCpf1_crRNA_array used for INT1_pos3, INT2_pos1 and INT3_pos1.
Figure S8. Pictures of transformants to demonstrate functionality of LbCpf1_crRNA_array for simultaneous genome editing at the INT1, INT2 and INT3 locus by introduction of donor DNA flank sequences and donor DNA carotenoid gene expression cassette sequences.
Figure S9. PCR results to determine correct integration of the *crtE* expression cassette in the INT1 locus, of the *crtYB* expression cassette in the INT2 locus and of the *crtI* expression cassette in the INT3 locus, using the single LbCpf1_crRNA_array.
Table S1. Primers used in this study.
Table S2. Donor DNA flanks and donor DNA expression cassettes nucleotide sequences.
Table S3. Overview of singleplex Cpf1 crRNAs and Cas9 control sgRNA used in this study.