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Genome editing systems across yeast species

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Yeasts are used to produce a myriad of value-added compounds. Engineering yeasts into cost-efficient cell factories is greatly facilitated by the availability of genome editing tools. While traditional engineering techniques such as homologous recombination-based gene knockout and pathway integration continue to be widely used, novel genome editing systems including multiplexed approaches, bacteriophage integrases, CRISPR-Cas systems, and base editors are emerging as more powerful toolsets to accomplish rapid genome scale engineering and phenotype screening. In this review, we summarized the techniques which have been successfully implemented in model yeast *Saccharomyces cerevisiae* as well as non-conventional yeast species. The mechanisms and applications of various genome engineering systems are discussed and general guidelines to expand genome editing systems from *S. cerevisiae* to other yeast species are also highlighted.

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Introduction

In modern biotechnology, yeast have been extensively studied due to advantageous traits including well established genetics, fast growth rate, simple nutrient requirements, have wider growth conditions compared to bacteria, lack of phage infectivity and many have been granted generally regarded as safe (GRAS) status by the US Food & Drug Administration [1]. Engineered yeast cell factories have been widely leveraged to produce recombinant proteins with post-translational modifications [2], pharmaceuticals [3], biofuels [4], fine chemicals [5,6] and other value-added products [7]. The development of industrially relevant yeast strains to meet the increasing demands of chemical production using cheap feedstocks is driven by the capability to modify yeast genome. Altering the carbon flux by tuning the expression level of metabolic pathway genes generally requires overexpression, deletion, or downregulation of specific genes.

Although the term 'genome editing' has been widely used, its definition remains to be clarified. In this review, we define genome editing as site-specific genome modification, inclusive of gene knockout, integration, and intentional point mutations. While traditional genome engineering techniques such as homologous recombination (HR) could efficiently achieve genomic modifications in baker's yeast *Saccharomyces cerevisiae* due to its dominant HR mechanism, this technique is much less efficient in other yeast species including *Yarrowia lipolytica* and *Pichia pastoris* where non-homologous end joining (NHEJ) is the major mechanism [8,9•]. The traditional iterative genome engineering approach, such as HR-based integration of cassettes, is hindered by the low integration efficiency resulting from low frequency of double-stranded DNA breaks, which significantly slows the design-build-test-learn (DBTL) cycle. To accelerate the engineering process across all yeasts, technologies such as the rapid-evolving clustered regularly interspaced short palindromic repeat (CRISPR)-mediated genome editing have emerged as a powerful toolbox in yeast metabolic engineering. While there are excellent reviews of yeast genome engineering [10–13], the rapidly evolving nature of the field warrants frequent updating. In this review, we will discuss various genome editing techniques (Table 1), highlight the recent advances of genome editing tools across biotechnologically important yeast species and give our perspectives on the future directions of yeast genome editing, with emphasis on how more advanced genome engineering tools might be adapted to non-conventional and non-model yeast.

Bacteriophage recombinases

Bacteriophage integrases were adapted for genetic engineering across species due to its high efficiency and specificity. Cre recombinase belongs to the tyrosine integrase family and is derived from the bacteriophage P1. The Cre-loxP system consists of the Cre recombinase and its substrate the loxP site. Efficient recombination between two loxP sites could lead to deletion, inversion, and translocation depending on the orientation of loxP sites (Figure 1a). An integration cassette with a selection marker flanked by loxP sites can be used for gene knockout and knock-in. For gene knockout process, the curing of the selection marker can be achieved by overexpression of the Cre recombinase. This system has been successfully adapted for metabolic engineering in *S.*

Table 1

Comparison of genome editing technologies in yeast

Technology	Mechanism	Advantages	Disadvantages
NHEJ	Non-homologous end joining	Random integration; Easy to implement	Variable expression level; Low efficiency for large fragments
Cre-loxP	Cre-mediated site-specific recombination	High efficiency; Easy to implement	Needs marker removal, loxP site left on genome. Lack of endogenous sites.
Serine integrase	Serine integrase mediated site-specific recombination	High efficiency; marker less integration; large integration capacity	Lack of endogenous sites for integration
TALEN	TALE nuclease mediated DSB	High specificity; programmable, multiplexing	Complex assembly of DNA construct
CRISPR-Cas	RNA guided nuclease mediated DSB	High efficiency, programmable, multiplexing, marker less integration	Possible off-target
Base editing	Deaminase fusion to nuclease deficient Cas9-mediated base conversion	No DSB; Programmable; Multiplexing	Often low efficiency; off-target effects underexplored.

cerevisiae [14,15], *Y. lipolytica* [16,17], *Kluyveromyces marxianus* [18] and *Ogataea polymorpha* [19]. Cre-loxP system was also implemented in the international synthetic yeast genome project, Sc2.0 to allow inducible genome rearrangement, presenting a powerful tool in synthetic biology [20]. One drawback of the Cre-loxP system is the loxP scar left on the genome after marker cure, which could lead to unintended genome alterations and gene losses through recombination between scars [21]. Because of the relatively low knock-in efficiency and lack of orthogonal Cre recombinases, the Cre-loxP system is generally used for single gene knockout.

Serine integrase is another type of phage integrase and has been developed to engineer certain non-model bacteria [22,23] which lack genetic tools. Serine integrase catalyzes the site-specific recombination between the attB and attP sites. Gene expression cassettes or whole plasmid can be integrated into host genome harboring the attachment sites via cassette exchange or single crossover, respectively (Figure 1b). Moreover, serine integrase-mediated *in vitro* recombination can be employed for rapid pathway assembly to accelerate the cloning process [24]. The high efficiency and capacity of this system make it an attractive tool for the genome engineering of yeast species. Xu *et al.*, investigated ten serine integrases in *S. cerevisiae* and identified a few highly active candidates suitable for genome engineering including ϕBT1, R4, BXB1, and ϕC31 [25••]. The efficiency of integrase-mediated recombination was ten times higher than that of HR while avoiding the repetitive scar sequences caused by Cre-lox. This study could be transferred to other yeast species to develop efficient genetic engineering tools.

TALENs

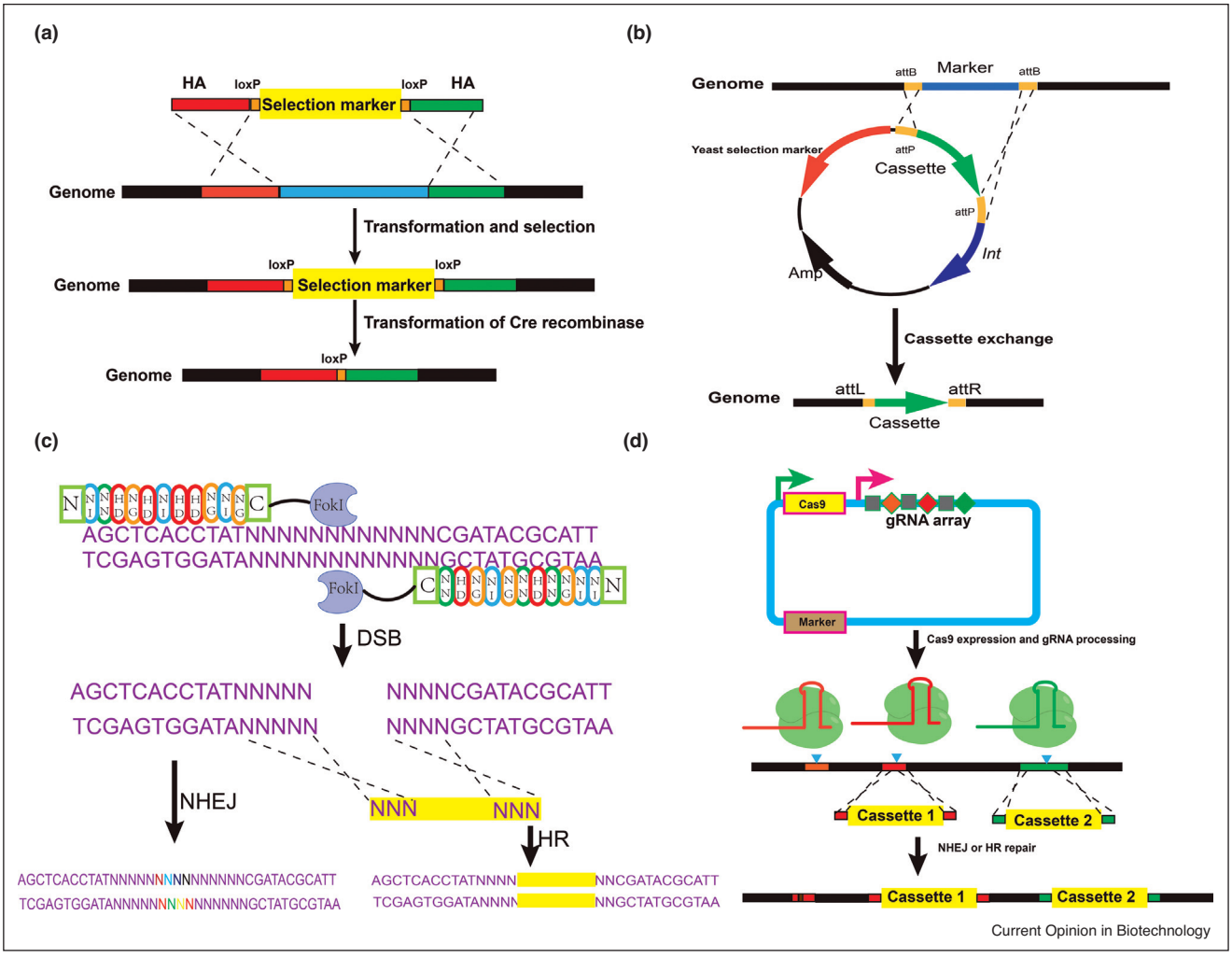
Transcription activator like effectors (TALEs) were first discovered in plant pathogen which exploits TALEs to infect its host. TALE Nuclease (TALEN) are TALEs with nucleases fused forming an artificial sequence-specific nuclease consisting of DNA binding domain of TALEs and the catalytic domain of FokI endonuclease [26,27]. The DNA-binding domain of TALENs consists of tandem repeats of 33–35 amino acids. Each repeat contains variable di-residues (RVDs) to determine the recognition of a single nucleotide [28] (Figure 1c). The modularity of TALENs allows assembly of construct to target essentially any DNA sequence. Efficient single gene knockout of *URA3*, *ADE2*, and *LYS2* based on NHEJ or homology directed repair (HDR) was reported in *S. cerevisiae* using a modular assembly strategy [29]. TALENs-mediated gene knockout and site-directed mutagenesis have been successfully implemented to enhance production of fatty acid in *S. cerevisiae* [30] and *Y. lipolytica* [31]. Zhang *et al.* designed a strategy based on TALENs-assisted multiplex editing (TAME) to accelerate the evolution of *S. cerevisiae* genome [32••]. In this work, targeting sequences for the TATA box and the GC box across the *S. cerevisiae* genome were identified using *in silico* scripts. TALENs were expressed under the inducible GAL promoter using mCherry as a reporter gene. Iterative induction and selection could identify mutants with the highest fluorescence. This strategy was then applied to evolve a yeast strain with faster glucose consumption and higher ethanol titer. The TAME screening was also leveraged to improve the stress tolerance to hyperosmotic pressure and high temperature in an industrial strain of *S. cerevisiae* [33]. TALENs have been largely used in *S. cerevisiae* despite its high efficiency for genome editing. Assembly of large repeats in TALENs is challenging which could limit its application in other yeast species especially when other genome editing systems such as CRISPR-Cas are available.

CRISPR-based technologies

CRISPR-Cas genome editing of S. cerevisiae

The type II CRISPR-Cas9 genome editor derived from the bacterial immune system has revolutionized biotechnology due to its high specificity and versatility. CRISPR-Cas9 systems cleave target DNA by the nucleoprotein

Figure 1



Schematic representation of yeast genome editing techniques. **(a)** HR and Cre-loxP system. Integration cassette with selection marker flanked by loxP sites is integrated into genome via HR. Selection marker is cured by expression of Cre recombinase. **(b)** Serine integrase-mediated marker less integration. Plasmid-containing serine integrase and integration cassette flanked by attP sites of serine integrase are transformed into yeast installed with attB sites of serine integrase. Expression of serine integrase could mediate recombination between attB and attP sites to integrase the cassette. **(c)** TALEN-mediated genome engineering in yeast. TALEN consists of a DNA-binding domain and a FokI nuclease domain. DSB is repaired by NHEJ to result in indel mutations or HR to achieve knock-in. **(d)** Multiplexed genome editing using CRISPR-Cas. Delivery of Cas effector and gRNA array can be achieved on a single plasmid. gRNA array is separated by a spacer such as tRNA to facilitate the gRNA processing. Simultaneous gene knockout and cassette knock-in is achieved.

complex formed between the Cas9 nuclease effector and an engineered single guide RNA (sgRNA) that cause double strand break (DSB) 3 bp upstream of the protospacer adjacent motif (PAM) sequence [34]. DSB are mainly repaired by NHEJ resulting in insertion or deletion (indel) mutations or by HDR (Figure 1d). The application of this system in model yeast *S. cerevisiae* was first described by DiCarlo *et al.* [35]. Expression of codon optimized Cas9 gene from *Streptococcus pyogenes* and guide RNA were delivered using two vectors. Toxicity of the CRISPR-Cas9 system was also observed when Cas9 and gRNA were simultaneously expressed. It was also found that co-transformation of a donor DNA with gRNA on a transient expression cassette or plasmid could significantly boost HR at the cutting site. The efficiency of single gene targeting in *S. cerevisiae* was generally high despite optimization of the design of gRNA is required. Currently, various web-based gRNA design algorithms were available to help minimize off-target effects [11]. Multiplexed genome editing is a desirable feature of the CRISPR-Cas system. Various strategies for delivery of multiple gRNA have been described. Bao *et al.* described the HI-CRISPR system where a Cas9 mutant with improved activity, crRNA array and tracrRNA were

expressed under different promoters on a high copy plasmid [36]. Pre-crRNA was processed into single spacer and formed functional complex with tracrRNA and *Sp*Cas9 protein. The efficiency of a triple deletion reached 87% using this system after four days of outgrowth in selective media. Mans and coworkers reported the introduction of up to six modifications to the *S. cerevisiae* genome including integration of multi-gene pathway, gene knock-out and site-directed mutagenesis in one transformation step [37]. In this work, up to two gRNAs which were designed by a web-based tool were carried on plasmids with different selection markers. It was found that six-gene deletion efficiency reached 65%. Horwitz *et al.* reported a technique for multiplexed gene deletion and pathway integration by using linearized plasmid bearing Cas9 and gRNA cassettes sharing homology with linearized plasmid in *S. cerevisiae* [38]. It was reported that the efficiency of triple deletion reached 64% and a 24-kb pathway for the biosynthesis of muconic acid could be integrated into three loci in one step transformation. Although multiple gRNA can be separately expressed, the cloning and sub-cloning process can be time-consuming. Fusion of self-cleaving hepatitis delta virus (HDV) ribozyme to gRNA to facilitate gRNA processing was able to achieve 86% and 43% targeting efficiency for duplex genome editing in haploid and diploid strain of *S. cerevisiae*, respectively [39]. Zhang *et al.* described a rapid multiplexed genome editing strategy by using an array of gRNA spaced by transfer RNA of glycine ($tRNA^{Gly}$) [40••]. The gRNA array was assembled using golden gate assembly, expressed under the SNR52 promoter and directly transformed into *S. cerevisiae*. It was reported that the disruption efficiency of eight genes reached 87%, which is the highest number of simultaneous gene deletions to date in *S. cerevisiae*. This strategy could be a valuable tool for rapid genome engineering of the baker's yeast.

Cas12a or Cpf1 was another CRISPR effector, belonging to the type V CRISPR system. Compared with Cas9, Cas12a recognizes distinct T-rich PAM sequence (TTTN) and is able to process the premature crRNA array, which is particularly suitable for multiplexed genome editing [41]. The CRISPR-Cas12a/Cpf1 system was also investigated for genome editing in *S. cerevisiae*. Verwaal *et al.* explored the functionality of three Cpf1 effectors: *Acidaminococcus* spp. Cpf1 (AsCpf1), *Lachnospiraceae bacterium* Cpf1 (LbCpf1) and *Francisella novicida* Cpf1 (FnCpf1) [42•]. The Cpf1 effectors were evaluated by targeted genomic integration of the three gene pathway of carotenoid biosynthesis and yellow fluorescent protein (YFP). It was found that LbCpf1 and FnCpf1 could reach targeting efficiency comparable to that of SpCas9. Multiplexed genomic integration of the carotenoid pathway into three non-coding regions was also achieved using LbCpf1 and a single crRNA array expressed by *SNR52* promoter. The efficiency of multiplexed editing was 91%. Li *et al.* developed a self-cloning CRISPR-Cpf1 system for genome editing and strain building in *S. cerevisiae* [43]. Single crRNA or crRNA array generated by PCR was co-transformed with a low copy plasmid bearing Cpf1 and a palindromic crRNA sequence. Complex of Cpf1 and palindromic crRNA will self-cleave the plasmid and allow the cloning of target crRNA via *in vivo* recombination. It was reported that efficiency of single and triplex genomic integration reached 80% and 32% using this self-cloning system.

Multiplexed automated genome scale engineering (MAGE) represents a powerful advance in yeast genome editing. The first report of yeast oligo genome engineering (YOGE) demonstrated that recombineering of yeast was possible but only about 1% efficient [44]. Soon after, eukaryotic MAGE (eMAGE) was reported. It relies on annealing of synthetic oligos to the lagging strand during DNA replication, and therefore, relies on the proximity the target site to the replication fork [45]. CRISPR-Cas9 was used to create double-strand DNA breaks at the δ-retrotransposon sites scattered throughout the genome, which facilitated substantially improved integration of large genetic circuits [46]. Thousands of specific single nucleotide genome edits were achieved using CRISPR-Cas9-and homology-directed-repeat (CHAnGE), where a library of homology repair templates encoding specific genomic changes is recombined at a double-stranded break caused by the CRISPR-Cas9. This genome-scale effort was demonstrated by selecting for gene disruptions causing tolerance to growth inhibitors [47]. Error prone PCR libraries of carotenoid pathway enzymes were introduced into the native enzyme genomic loci by CRISPR-Cas9-mediated double strand break and homology directed repair. This method of Cas9-mediated protein evolution reaction (CasPER) resulted in an 11-fold improvement in isoprenoid production and demonstrating efficient enzyme directed evolution in a genomic context [48].

Genome editing in non-conventional yeasts using CRISPR-Cas

In nearly all the aforementioned works, the high efficiency of homology directed repair in *S. cerevisiae* was utilized. Most non-conventional yeasts are highly inefficient at homology directed repair compared with non-homologous end-joining. As a result, different approaches for genome editing are needed.

Yarrowia lipolytica

The oleaginous yeast *Y. lipolytica* has been extensively studied in recent years due to its capability to accumulate large amounts of lipids [49–51], and ability to grow on a wide range of substrates including xylose [52], acetic acid, fatty acids, and alkanes [53]. *Y. lipolytica* has been engineered to produce value-added chemicals including β-carotene [54,55], polyketide [56,57], polyunsaturated

fatty acids [5] and terpenoids [58,59]. The CRISPR-Cas system has been adapted to facilitate metabolic engineering in *Y. lipolytica* [60]. Despite the high efficiency of CRISPR-Cas9 genome editing in *S. cerevisiae*, implementing this system in *Y. lipolytica* required non-obvious suitable promoters for the expression of gRNA. Schwartz *et al.* reported genome editing of *Y. lipolytica* using CRISPR-Cas9 for the first time [61••]. The authors optimized the expression of gRNA using both RNA polymerase type II promoter (TEF promoter) and several predicted RNA polymerase type III promoters. It was found that a synthetic SCR1′-tRNA^{Gly} promoter could achieve the highest editing efficiency (92%) of *PEX10* gene after outgrowth. Quantitative PCR analysis of the abundance of gRNA under different promoters revealed that the level of gRNA transcript under SCR1′-tRNA^{Gly} promoter was high but not the highest, indicating that the editing efficiency may not be positively correlated with transcript level. This work also highlights outgrowth in selective media can significantly boost the gene editing efficiency. Using this CRISPR system, Schwartz *et al.* also identified 5 out of 17 loci for efficient markerless genomic integration in NHEJ capable cells [62•]. Stable integration can be achieved by transforming the CRISPR-Cas9 plasmid and the respective donor plasmid harboring the desired gene cassette. Holkenbrink *et al.* developed a streamlined toolbox EasyCloneYALI which consists of a set of vectors for efficient genome integration and gene knock-out in *Y. lipolytica* via CRISPR-Cas9 in NHEJ deficient KU70 knockouts [63••]. It was reported that up to 80% of integration efficiency, 90% efficiency of single gene knock-out and 66% efficiency of double knock-out, can be achieved.

To establish a multiplexed genome editing strategy for *Y. lipolytica*, Gao *et al.* constructed a single plasmid to overexpress Cas9 and multiple gRNA under the TEF intron promoter [64]. Self-cleaving hammer head ribozyme (HHR) and HDV were added to flank the gRNA sequence to facilitate the release of mature gRNA. It was observed that expression of two gRNA on a single plasmid outperforms that on two separate plasmids in terms of duplexed editing efficiency. The efficiency for double knock-out and triple knock-out was 36.7% and 19.3%, respectively. It should also be noted that outgrowth was performed to achieve the high editing efficiency.

In a recent study, the CRISPR-Cas12a system was adopted for efficient and multiplexed genome editing in *Y. lipolytica* [65•]. Yang *et al.* implemented this system by overexpressing the AsCas12a gene and gRNA under various promoters. It was observed that the highest editing efficiency (93.3%) for targeting *CAN1* was achieved under the TEF intron promoter without adding any HHR or HDV, which indicates that TEF intron promoter could be a promising candidate for the expression of self-cleaving crRNA. Duplexed and triplexed genome editing was also achieved by overexpressing multiple crRNA on a single plasmid, reaching an efficiency of 83.3% and 41.5%, respectively. A second study of Cas12a activity in *Y. lipolytica* focused on gRNA engineering, demonstrating high efficiency disruptions using 23–25 bp gRNAs that could be multiplexed with truncated gRNAs that caused robust gene repression [66].

CRISPR-Cas9 was leveraged to study functional genomics of *Y. lipolytica* by constructing a genome-wide gRNA library to target 7845 coding sequences (CDS) [67••]. Six gRNAs were designed to target each CDS. Three *Y. lipolytica* strains: Po1f, Po1f harboring Cas9 and Po1fΔ*ku70* harboring Cas9 were used to validate the gRNA library. Fitness score (FS) and cutting score (CS) were defined to evaluate the essentiality and cutting efficiency of each gRNA by measuring the abundance of gRNA in strain Po1f-Cas9 and Po1fΔ*ku70*-Cas9 relative to that in control Po1f strain. CS values were used to identify efficient gRNAs and exclude inactive gRNAs from essential gene determination and to obtain a validated library. A total of 1377 CDSs were identified as essential based on the validated library. This library was also a useful tool to facilitate screening of growth and non-growth associated phenotypes such as canavanine resistance and lipid content screening.

Rhodosporidium toruloides

Another oleaginous yeast species *R. toruloides* can accumulate high content of lipids and carotenoids. It can also covert lignocellulose hydrolysate into valuable compounds [68], making this yeast an attractive platform to produce acetyl-CoA derived chemicals using renewable feedstocks. CRISPR-Cas9 genome editing of *R. toruloides* was achieved by integrating a cassette to express Cas9 under GAP promoter and gRNA under *S. cerevisiae* SNR52 promoter [69•]. Targeting efficiency of *URA3* was estimated to be 0.001%. Further optimization including gRNA target sequence and the use of tRNA^{Phe} promoter improved editing efficiency of *URA3* to 0.62%. Duplexed editing of *URA3* and *CAR2* was achieved by expressing two gRNAs for each target gene in an array spaced by tRNA^{Gly}, with efficiency of 30%. In another study, codon optimized Cas9 of *Staphylococcus aureus* was first integrated into the *R. toruloides* genome. Two U6 promoters were identified to drive the expression of gRNA. It was observed that editing efficiency of *CRT1*, *CAR2*, and *CLYBL* achieved 66.7%, 75%, and 75%, respectively. The HR-mediated knockout of *CRT1* was also explored with an efficiency of 8%, likely due to the NHEJ dominant repair mechanism [70•]. Schultz *et al.* optimized the expression of SpCas9 under various Pol II promoters and gRNA using five different promoter constructs in *R. toruloides* [71••]. The expression of SpCas9 driven by PGK1 promoter and gRNA by a 5S-tRNA^{Gly} fusion promoter led to the highest editing efficiency of

CRTYB (99%), *CRTI* (96%), and *LEU2* (88%). Duplexed editing of *CRTYB* and *LEU2* was also achieved with a double knockout efficiency of 78%. This work highlighted a strategy to optimize promoters for the expression of gRNA in non-conventional yeasts.

Schizosaccharomyces pombe

The fission yeast *S. pombe* is gaining research interests one of the model organisms for genetics and cellular biology [72]. To unlock the research and industrial potential of this yeast, genetic engineering tools based on CRISPR-Cas9 has been developed. Jacob *et al.* described a strategy by placing gRNA between the *rrk1* promoter and a cleavable leader sequence and HHR. Transformation of gRNA construct and Cas9 on two plasmids to target *ADE6* led to efficient disruption of this gene (85%–98%) when a donor cassette was co-transformed [73]. The same strategy was implemented in *S. pombe* by using a fluoride counter-selectable marker [74]. Targeting efficiency of 33% was obtained for *PIL1*. Zhang *et al.* described a cloning-free procedure by exploiting the gap repair mechanism in *S. pombe*. High knock-in efficiency (84%) was achieved by co-transforming a 90 bp donor oligo [75]. Hayashi and coworker described genome editing in *S. pombe* using microhomology-mediated end-joining (MMEJ) [76]. It was found that co-transformation of Cas9/gRNA plasmid with short oligos (15 bp, 20 bp, or 25 bp) lead to point mutation (W104A) of *swi6* gene with efficiency of 10%–100%. The effectiveness of this strategy was also validated by introducing point mutation (S604A) to the *mrc1* gene (encoding mediator of replication checkpoint protein 1) with 70% knock-in efficiency. In a recent study, CRISP-Cas12a was implemented in *S. pombe* by expressing FnCas12a under *adh1* promoter and crRNA under *rrk1* promoter in a single plasmid [77•]. The targeting efficiency of *ADE6* was 25–50% (10–30% for *LEU1*, *HIS3*, and *LYS9*) using a HR donor DNA with 800 bp homology to the ends of DSB. The authors also investigated other endogenous pol II promoters to express crRNA and found that the *fba1* promoter can result in 30% higher editing efficiency of *ADE6*. Duplexed (ADE6 and LEU1) and triplex (*ADE6*, *LEU1*, and *HIS3*) editing was also achieved using crRNA array expressed by the *fba1* promoter, reaching efficiency of 17% and 2.5%, respectively.

Komagataella phaffii (previously Pichia pastoris)

The methylotrophic yeast *K. phaffii* has been recognized as an industrially relevant workhorse to produce recombinant proteins. Lack of episomal plasmids and genetic engineering tools has hindered metabolic engineering of this promising host. Weninger *et al.* optimized CRISPR-Cas9 genome editing in *K. phaffii* by comparing the cutting efficiency of *GUT1* [78]. It was found that the RNA polymerase III promoters tested led to poor editing efficiency (0–31.7%) while the HHR-gRNA-HDV construct using TEF promoter achieved much higher cutting efficiency (87–94%). Duplexed editing efficiency of *GUT1* and *AOX1* achieved 69% when human codon optimized Cas9 was expressed under the HXT1 promoter and gRNA with the highest efficiency was used. In another work published by Weninger *et al.*, CRISPR-Cas9-mediated HDR was investigated in a *KU70*-deficient *K. phaffii* strain [79]. When a marker-free linear donor cassette was co-transformed with a CRISPR plasmid targeting *GUT1*, integration efficiency of 78%–91% was obtained, which was 1.9-fold higher than that of the control (without CRISPR plasmid). It was also found that HDR efficiency was improved in wild type *K. phaffii* strain when the donor cassette was supplied on a circular or linearized plasmid containing an autonomous replicating sequence (ARS). Delivery of Cas9 and gRNA on an episomal plasmid to achieve genome editing in *K. phaffi* was also reported recently [80]. Editing efficiency of *GUT1* was comparable to that obtained when Cas9 and gRNA was integrated. To achieve multiple genomic integration in *K. phaffii*, Liu *et al.* screened ten potential loci using an enhanced green fluorescent protein (eGFP) cassette in a *KU70* knock-out strain [81]. gRNAs with the highest targeting efficiency for each locus were identified. Three linear cassettes containing eGFP, mCherry and blue fluorescent protein (BFP) were combinatorically transformed with corresponding gRNA to achieve multi-loci integration. It was observed that efficiency of 57.7%–70%, 12.5%–32.1% was obtained for double and triple integration, respectively. Yang and coworkers reported a CRISPR-Cas9 system in *K. phaffii* by genomic integration of codon optimized SpCas9 and episomal expression of gRNA flanked by HHR and HDV under the bidirectional HTX1 promoter [82•]. It was found that the editing efficiency could reach 80%–95% when six genes were targeted using this system. Duplexed gene editing was also tested using this system by expressing two gRNAs under the HTX1 promoter. However, zero to very low double knockout efficiency was observed.

Scheffersomyces stipitis

S. stipitis has gained increasing interest as a promising host for biomanufacturing due to its ability to consume xylose. Implementing a plasmid-based CRISPR-Cas9 system in *S. stipitis* required a stable episomal vector to deliver Cas9 and gRNA. This issue was addressed by identifying centromeric DNA sequence to help stabilize episomal plasmid [83••]. Successful gene editing of *ADE2* and *TRP1* was achieved by expression of codon optimized Cas9 and gRNA under the SNR52 promoter on the engineered episomal plasmid. This system was then expanded to marker-less donor integration using HDR in a *S. stipitis KU70* and *KU80* double mutant strain [84]. It was found that donor DNA knock-in efficiency reached 73 % to 83% when homologous arms (HAs) of various lengths (50 bp, 100 bp, 500 bp, and 1 kb) were used.

Kluyveromyces lactis and K. marxianus

CRISPR-Cas9 genome editing of *K. lactis* and *K. marxianus* was first achieved by overexpressing the Cas9 under TEF promoter and gRNA flanked by HHR and HDV under TDH3 promoter of *S. cerevisiae* [85••]. Targeting efficiency of *ADE2* was found to be 54% and 100% in *K. lactis* and *K. marxianus*, respectively. It was also found that co-transformation of a donor DNA could improve the targeting efficiency in *K. lactis* to 96%. PCR analysis revealed some editing events were repaired by the donor DNA. This system was also highly efficient in diploid strain of *K. marxianus*, reaching 80% of editing efficiency of *ADE2*. Marker-less donor integration in *K. marxianus* was reported in another study [86]. Native SNR52 promoter of *K. marxianus* was employed to express gRNA. Donor cassette with 50 bp HAs was able to integrate into the *URA3* locus and *SED1* locus with an efficiency of 92% and 100% in a NHEJ-deficient strain. Lee *et al.* implemented the CRISPR-Cas9 system in *K. marxianus* by integrated expression of Cas9 under Lac4 promoter and gRNAs under SNR52 promoter [87]. Successful knockout of MATa3 gene was obtained using this system. Optimization of hybrid promoters for the expression of gRNAs in *K. marxianus* was performed in another report [88•].It was found that the highest targeting efficiency (66%) of XYL2 (encoding xylitol dehydrogenase) was achieved using RPR1-tRNA^{Gly} promoter. This system was employed to disrupt seven alcohol dehydrogenase genes (ADH1-7) and alcohol-O-acetyltransferase gene (ATF) with targeting efficiency of 10%–67%.

O. polymorpha (previously Hansenula polymorpha)

O. polymorpha is a thermo-tolerant methylotrophic yeast widely used to produce recombinant proteins. It is also endowed with ability to utilize a broad range of substrates including glycerol, glucose, xylose, and cellobiose [89]. Numamoto *et al.* reported the editing of *ADE12*, *PHO1*, *PHO11* and *PHO84* using tRNA^{CUG} as promoter to express gRNA [90]. Targeting efficiency of 45% was reached for *ADE12* while more modest editing efficiency (17–71%) was obtained for *PHO1*, *PHO11* and *PHO84*. Juergens *et al.* implemented a different strategy to target *ADE2* by using an HHR-gRNA-HDV fusion construct under the TDH3 promoter. It was found that outgrowth in liquid culture was needed to achieve observable cutting of *ADE2*. Cutting efficiency of *ADE2* reached 31% and 61% after 48 hour and 96 hour of outgrowth, respectively. Further outgrowth did not improve the editing efficiency. Duplexed editing of *ADE2* and *YNR1* was performed by expressing an array of HHR-gRNA-HDV under the TDH3 promoter, reaching double knockout efficiency of 2%–5%. To establish a robust multiplexed genome editing tool in *O. polymorpha*, Wang *et al.* integrated the Cas9 expression cassette into the genome. The gRNA construct was expressed by SNR52 promoter of *S. cerevisiae* and integrated the *ADE2* locus to facilitate the visual selection of positive red colonies [91]. Both expression cassette of Cas9 and gRNA can be excised by transforming proper linear DNA cassettes to allow iterative genome editing. Editing efficiency of *LEU2* and *URA3* reached 58% and 65%, respectively. Triple gene knockout of *URA3*, *HIS3*, and *LEU2* by transforming a cassette of gRNA array was achieved with efficiency of 23.6%. Integration of the three gene resveratrol pathway into the *URA3*, *HIS3* and *LEU2* was obtained with efficiency of 30.5% by co-transforming three donor cassettes. Multi-copy integration of GFP and resveratrol pathway into the rDNA site was also achieved by targeting the rDNA region. It was found that up to 9.8 copies of the genes were integrated into the rDNA sites, leading to improved titer of resveratrol.

Issatchenkia orientalis

I. orientalis is a non-conventional yeast which has been exploited to produce organic acids such as succinic acid [92] due to its tolerance to low pH. Efforts have been made to enable metabolic engineering of this promising host by the development of a CRISPR-Cas9-based gene knockout tool [93••]. Similar to other non-conventional yeasts, lack of stable episomal plasmid needs to be addressed to allow expression of Cas9 and gRNA. Tran *et al.* constructed a plasmid based on the *S. cerevisiae* ARS to achieve stable expression of GFP in this yeast. Suitable promoter for gRNA expression was identified by screening a series of RNA polymerase III promoters to target *ADE2* gene. It was observed that the highest cutting efficiency was obtained using a hybrid RPR1′-tRNA^{Leu} promoter. Targeting efficiency of single gene knockout (*LEU2*, *HIS3* and *TRP1*) reached near 100%. Triplexed editing of *ADE2*, *HIS3*, and *SDH2* was achieved with 46.7% efficiency. Cao *et al.* reported a suite of genetic toolbox for rapid metabolic engineering in *I. orientalis* by identifying centromeric DNA sequence to enhance gene expression from episomal plasmid, characterizing various promoters, terminators, and *in vivo* DNA assembly [94].

Candida tropicalis, Candida albicans

C. tropicalis isa pathogenic yeast which can accumulate high titer of long chain dicarboxylic acid [95]. A CRISPR-Cas9 genome editing system was recently developed for *C. tropicalis* [96]. Codon optimized *Sp*Cas9 was expressed under the GAP1 promoter and HHR-gRNA-HDV was expressed under the FBA1 promoter. Both integrated and transient CRISPR-Cas9-gRNA cassette can efficiently target the *URA3* gene when a donor DNA with 50 bp homologous arms was supplied. It was also found that combining donor DNA and the CRISPR-Cas9-gRNA in one cassette significantly enhanced the editing efficiency of *ADE2* than a separate donor DNA. Duplexed editing of *URA3* and *ADE2* was achieved by transforming a cassette with Cas9, two gRNAs and two donor DNA, obtaining an efficiency of 32%.

C. albicans is human pathogen which can cause fatal infections. Genome editing of this diploid yeast species could accelerate the discovery of potential antifungal drugs. Vyas *et al.* described a CRISPR system by expressing codon optimized Cas9 from ENO1 promoter and gRNA from SNR52 promoter on an integrated cassette [97]. This system was able to efficiently target various genes including *ADE2*, *URA3*, *RAS1*, *MtlA1*, and *TPK2*. It was also found that this CRISPR-Cas9 system could target genes with homology such as CDR1 and CDR2 with 20% efficiency. Transient expression of Cas9 and gRNA was also able to target *ADE2* when a donor cassette was supplied [98]. Nguyen developed a rapid and recyclable CRISPR system by using leucine auxotrophy or the FLP-FRT system to excise the Cas9 and gRNA cassette from *C. albicans* genome [99].

Yeast genome editing using base editors

CRISPR-Cas-based genome editing generates DSB to modify target DNA. Base editing is a novel genome editing technology to introduce point mutations without DNA cleavage in non-dividing cells, which has great potential in genome editing and gene therapy. Catalytically deficient Cas9 effector is employed to recruit gRNA to the target locus. Deaminase fused to dCas9 converts cytidine to uridine or adenine to guanine on the noncomplementary strand [100]. Base editors function in an activity window, meaning that all cytidine bases within this window could be targeted. To reduce the off-target effect of base editor, Tan *et al.* implemented two strategies by engineering the rigidity of linker between the dCas9 domain and the deaminase domain and fusion of truncated deaminase to dCas9 [101]. Target-AID base editor was constructed in *S. cerevisiae* by using a protein complex of dCas9 and PmCDA1 [102]. It was found that expression of dCas9-PmCDA1 or nCas9-PmCDA1 could mediate efficient point mutation of *CAN1* and *ADE1*. Efficiency of double editing of CAN1 and ADE1 was found to be 31%. Recently, the Target-AID base editor was adapted in *Y. lipolytica* by using the nCas9-pmCDA1 fusion protein [103••]. *TRP1* gene was targeted by introducing a nonsense mutation. It was found that no mutation was observed when nCas9-pmCDA1 or nCas9-pmCDA1-UGI was expressed. Outgrowth of 24 hour was performed and base editing occurred only in the *KU70* knockout strain with efficiency of 28% after 96 hour of growth. It was also revealed that deletion of *KU70* can improve the base editing accuracy by installing the desired mutation while more indel mutations occurred in the wild type strain. Multiplexed base editing was also achieved by expressing multiple gRNAs and base editor on a single plasmid. It was reported that duplexed base editing of *TRP1* and *PEX10* achieved efficiency of 31% after 96 hour of outgrowth while triple editing was not observed. Despite base editing is less commonly used compared with regular CRISPR-Cas genome editing, it could be a useful supplementary genome editing tool due to its simplicity, DSB-free and donor-free procedure.

Emerging CRISPR technologies for yeast genome editing

Base editors can introduce four types of transition mutations. However, transversion mutations such as adenine to thymidine are needed to correct sickle cell disease (SCD). Recently, a novel base editing technology termed prime editing which was able to perform all 12 possible base-to-base mutations, insertions, and deletions without DSB or donor DNA was reported [104]. Prime editors consist of prime editing guide RNA (pegRNA), Cas9 nickase (nCas9) fused to an engineered reverse transcriptase (RT). The pegRNA contains a sgRNA that directs nCas9 to the target site and primer-binding site (PBS) which includes new genetic information and hybridizes with the PAM strand. Nicking of the PAM strand and hybridization between PBS and PAM strand will initiate reverse transcription to directly install the edit into the target site. The authors developed three prime editors: PE1, PE2 and PE3. In PE1, wild-type Maloney murine leukemia virus (M-MLV) RT was fused to nCas9 and detectable editing was observed. To enhance the editing efficiency, five mutations were introduced to enhance the thermostability, processivity and DNA-RNA substrate affinity of M-MLV RT. The engineered RT was used in PE2 and improved the editing efficiency by 5.1-fold compared with PE1. In PE3, a second sgRNA was used to nick the non-target strand to direct DNA repair machinery to replace the original strand with the edited strand. This further enhances the editing efficiency by 4.2-fold compared with PE2. The prime editors described here could be a powerful and versatile genome editing toolbox for yeast genome editing in the future.

Comparison of genome editing systems

Various yeast genome editing systems were discussed in this review and a comparison of the yeast genome editing systems was summarized in Table 1. In most yeasts where NHEJ is the dominant repair mechanism, construction of mutant library via NHEJ is a viable strategy due to the random repair in yeast genome. However, HR is preferred to NHEJ as HR offers a more precise and predictable genome engineering approach for rational metabolic engineering. Non-Cas9-mediated HR is still widely employed to disrupt genes and integrate metabolic pathway into yeast genome, despite the low integration efficiency for large size DNA cassettes. HR remains the go-to technique particularly for non-conventional yeast when other genome editing tools are not readily available. Knockout of *KU70* or *KU80* gene is a common and effective strategy to enhance HR efficiency not by increasing HR rates but by decreasing NHEJ rates. Serine integrase-mediated integration is mainly exploited as a tool to integrate large cassettes into genome; however, these methods still require integration of the attachment sites. Serine

integrases could also be used to achieve high throughput DNA assembly and integration in single transformation step. TALEN could be used for multiplexed and genome scale engineering; however, its application in yeast genome engineering may be hindered by the cost and complexity of DNA assembly of TALEN. CRISPR-Cas-based genome editing is showing great promise in yeast genome engineering due to its programmability and multiplexing. Design of efficient guides and assembly of gRNA array to target large number of genes are still the main challenge that needs to be addressed. Given the natural processing of Cas12a gRNA arrays, it may be preferred for multiplex applications. Base editing methods, including prime editing, are well suited for making point mutations, such as introduction of stop codons.

Perspectives

Yeast has become a promising chassis to address challenges in growing demand of a sustainable economy. Construction of cost-effective yeast cell factories is driven by the development of genome editing tools. CRISPR-Cas system has been successfully implemented mainly in genome engineering of *S. cerevisiae*. Development of CRISPR-Cas genome editing system in other non-conventional yeasts was bottlenecked by the lack of suitable promoters for the expression of Cas9 and gRNA. According to literature, a strong promoter typically drives the expression of Cas9, and increasingly. tRNA and hybrid RNA polymerase II gene promoters are successful for gRNA processing and multiplexing.

Multiplexed genome editing would be accelerated by rapid assembly of multiple gRNAs [105,106]. CRISPR systems using orthogonal Cas effectors to simultaneously achieve gene activation, gene repression and gene deletion have been described [107]. This strategy could be a promising approach to achieve high throughput metabolic engineering of yeast cell factories. Engineered Cas effector variants with improved genome editing efficiency, reduced off-target effect [108] and altered PAM preference [109] could be expanded to yeast genome editing to facilitate strain engineering. Cas12a is likely to be more used for multiplex genome engineering.

Given the increasing interest in non-conventional yeast in biotechnology, a further expansion of genome editing tools will be needed; however, direct translation from *S. cerevisiae* into these systems is unlikely due to differences in the propensity for DSB repair by NHEJ. Therefore, we expect that genome engineering strategies that don't rely on HDR, such as serine integrases, will begin to play a more central role, especially when combined with genome scale libraries and larger metabolic pathways.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Zhiliang Yang: Conceptualization, Writing - original draft, Writing - review & editing. **Mark Blenner:** Conceptualization, Writing - original draft, Writing - review & editing.

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