Engineering Microbiology 3 (2023) 100094

Contents lists available at ScienceDirect

Engineering Microbiology

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

Genetic tools for metabolic engineering of *Pichia pastoris*

Xiaoyan Wu[a,b,c], Peng Cai[a,b], Lun Yao[a,b,d], Yongjin J Zhou[a,b,d,*]

[a] Division of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Dalian Key Laboratory of Energy Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of7 Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

ARTICLE INFO

Keywords:
Pichia pastoris
Genetic tools
Metabolic engineering
CRISPR/Cas9

ABSTRACT

The methylotrophic yeast *Pichia pastoris* (also known as *Komagataella phaffii*) is widely used as a yeast cell factory for producing heterologous proteins. Recently, it has gained attention for its potential in producing chemicals from inexpensive feedstocks, which requires efficient genetic engineering platforms. This review provides an overview of the current advances in developing genetic tools for metabolic engineering of *P. pastoris*. The topics cover promoters, terminators, plasmids, genome integration sites, and genetic editing systems, with a special focus on the development of CRISPR/Cas systems and their comparison to other genome editing tools. Additionally, this review highlights the prospects of multiplex genome integration, fine-tuning gene expression, and single-base editing systems. Overall, the aim of this review is to provide valuable insights into current genetic engineering and discuss potential directions for future efforts in developing efficient genetic tools in *P. pastoris*.

1. Introduction

Pichia pastoris is a generally regarded as safe microorganism that has been widely used as a platform for both fundamental research and industrial applications in recombinant protein expression [1,2]. *P. pastoris* is a Crabtree negative yeast, which does not accumulate by-products such as ethanol, making it a promising chassis microbe for chemical production with potential for high yields [3]. Additionally, as a methylotrophic yeast, it is being increasingly explored as a host for methanol biotransformation [4,5]. *P. pastoris* has demonstrated potential for the production of a wide range of products, including terpenes [6–8], polyketides [9,10], flavonoids [11], catharanthine [12], fatty acid derivatives [13], and 3-hydroxypropionic acid [14].

Genomics [15–18], transcriptomics [19] and proteomics [20] studies have provided valuable insights into the cellular metabolism of *P. pastoris*. Furthermore, several genome-scale metabolic models such as PpaMBEL1254 [21], iPP668 [22], and iLC915 [23] have been developed to design strategies for rewiring cellular metabolism for the production of target compounds. To achieve this, proficient genetic engineering tools such as promoters, terminators, plasmids, and gene editing systems are essential. Fortunately, advanced gene-editing tools such as the Cre/*Lox* recombination [24] and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system [25], have been developed, greatly facilitating metabolic engineering efforts in *P. pastoris*.

This review discusses recent advances in developing genetic tools in *P. pastoris*, including promoters and terminators, plasmids, genome integration sites, and genetic editing systems. In particular, prospects and challenges in the development of multiplex genome engineering tools, gene regulation systems, and single-base editing systems are also discussed, providing guidance for future developments of efficient genetic tools in *P. pastoris*.

2. Promoters and terminators

A promoter is a DNA sequence located upstream of a gene that regulates transcription by RNA polymerase and transcription factors [26]. In eukaryotes, promoters are more complex than those in prokaryotes, and are typically categorized into core promoters and regulatory components [27]. The core promoter determines the transcriptional level and basic promoter strength, while the regulatory components consist of an upstream activating sequence (UAS) or upstream repressing sequence (URS), contain one or more transcription factor binding sites (TFBSs) that activate or repress transcription by binding to specific transcription factors (TFs) (Fig. 1A). Promoters play a pivotal role in directly controlling gene expression levels. Therefore, the characterization and construction of inducible and constitutive promoters of variable strengths is critical for rewiring cellular metabolism and optimizing biosynthetic pathways [28,29]. To provide a promoter toolbox for

* Corresponding author.
E-mail address: zhouyongjin@dicp.ac.cn (Y.J. Zhou).

https://doi.org/10.1016/j.engmic.2023.100094
Received 14 January 2023; Received in revised form 11 May 2023; Accepted 11 May 2023
Available online 14 June 2023

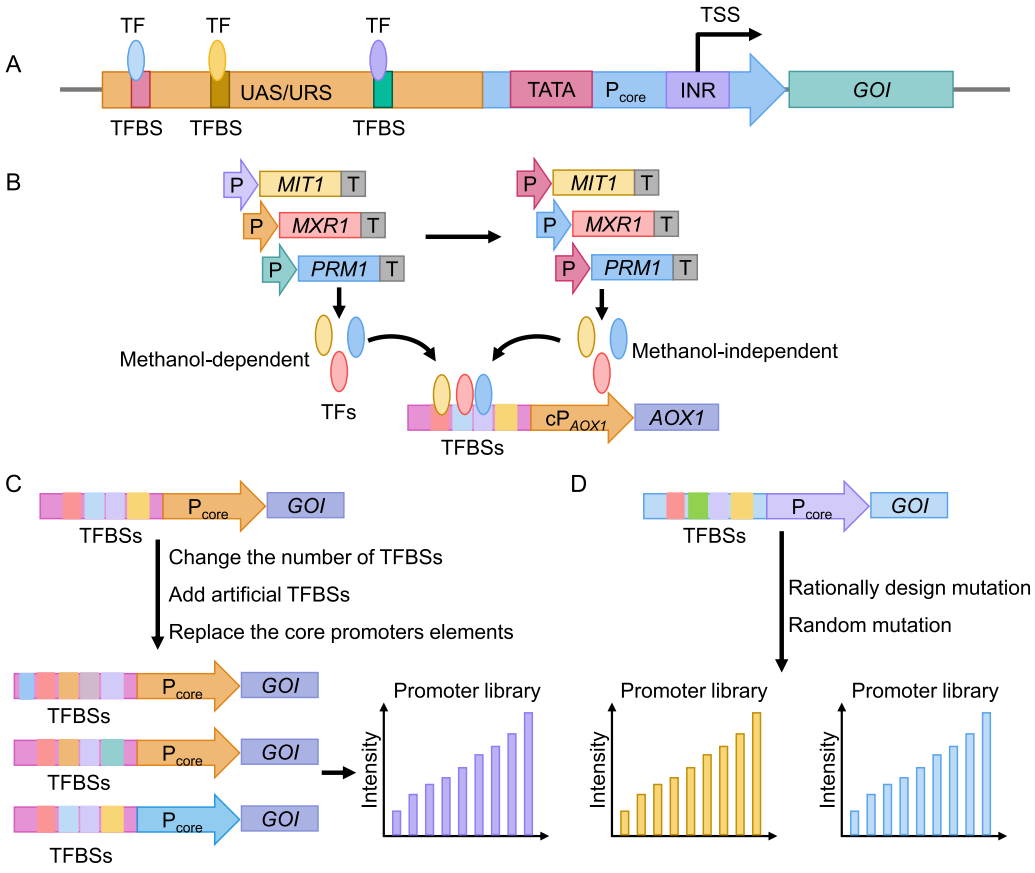

Fig. 1. Overview of promoter engineering strategies in *P. pastoris*. (A) Structure of promoter. TF: transcription factor, TFBS: transcription factor binding site, UAS: upstream activating sequence, URS: upstream repressing sequence, INR: initiator, TSS: transcriptional start site, *GOI*: gene of interest. (B) Alteration of P_{AOX1} expression performance from methanol-dependent to methanol-independent. (C) Regulating promoter strengths by changing TFBSs and core promoter sequences. (D) Promoter libraries were constructed by altering promoter strength through mutation.

metabolic engineering in *P. pastoris*, Vogl et al. characterized 45 promoters involved in the methanol utilization pathway under different carbon source conditions (glucose or methanol) using high-throughput 96-deep-well plates [30]. We also evaluated the expression patterns of 18 promoters of *P. pastoris* across various carbon sources (methanol, glucose, and methanol/glucose mixture) in shaken flask culture [31]. Table 1 lists the most commonly used promoters in *P. pastoris* for metabolic engineering.

The promoter of the alcohol oxidase I (*AOX1*) gene (P_{AOX1}) is considered the strongest inducible promoter and is frequently utilized for recombinant protein production in *P. pastoris* [32]. It is strongly induced by methanol but repressed by glycerol, ethanol, and glucose, with its activity being regulated by interactions between TFs and their TFBSs, also known as *cis*- and *trans*-acting elements. The *cis*-acting elements of P_{AOX1} are located in the D (−638 and −510) and E (−552 and −442) regions [33]. Three transcriptional activators Mit1, Mxr1, and Prm1, respond to methanol and activate the promoter in a cascade without interfering with each other [34]. Mxr1 mainly functions during carbon de-repression, while Mit1 and Prm1 function during methanol induction. Three transcriptional repressors Nrg1, Mig1, and Mig2, can bind to P_{AOX1} and inhibit its expression in the presence of glucose or glycerol [35,36]. Nrg1 competes with Mxr1 for P_{AOX1} binding sites, as Nrg1-deficient strains can express Aox1 at low glucose concentrations. To enable expression under P_{AOX1} in a methanol-independent manner, the expression of Mit1, Mxr1, and Prm1 under the derepressed promoter P_{CAT1} [37], or the expression of Mxr1 under the weak methanol-induced promoter P_{AOX2} [38] can be utilized (Fig. 1B). Changing the sequence of P_{AOX1} is another feasible approach to regulate the promoter strength.

Table 1
The commonly used promoters in *P. pastoris*.

Promoter	Type	Expression level	Ref.
P_{AOX1}	Methanol inducible	Strong[a]	[30]
P_{DAS1}	Methanol inducible	Strong	[30]
P_{DAS2}	Methanol inducible	Strong	[30]
P_{PMP20}	Methanol inducible	Strong	[30]
P_{CAT1}	Methanol inducible	Strong	[30]
P_{FDH1}	Methanol inducible	Strong	[30]
P_{FLD1}	Methanol inducible	Strong	[30]
P_{AOX2}	Methanol inducible	Weak	[30]
P_{GTH1}	Limited glucose inducible	Strong[b]	[135]
P_{THI11}	Repressed by thiamine	Strong	[50,136]
P_{GAP}	Constitutive	Strong	[30,32]
P_{TEF1}	Constitutive	Strong	[32]
P_{GCW14}	Constitutive	Strong	[54]
P_{PDC}	Constitutive	Strong	[137]
P_{ADH3}	Constitutive	Intermediate	[138,139]

[a] Methanol inducible: strong, >67%; intermediate, 33–67%; weak, <33% of P_{AOX1}.
[b] Other types: strong, >67%; intermediate, 33–67%; weak, <33% of P_{GAP}.

At least 12 *cis*-acting elements have been identified in P_{AOX1}, and deletion or replication of putative TFBSs has generated a promoter library ranging from 6% to 160% of wild-type P_{AOX1} activity [39]. Replacing the native P_{AOX1} core promoter with synthetic ones resulted in expression strengths of 10% to 117% of P_{AOX1}, with preserved glucose inhibition and methanol induction (Fig. 1C) [40]. Fusing a synthetic core

promoter to the *cis*-regulatory module of P_{AOX1} generated a promoter library with various strengths (0.3% to 70.6% of wild-type P_{AOX1}) in *P. pastoris* [41]. Substituting specific *cis*-regulatory DNA elements with synthetic triplet-Cat8 and Adr1 *cis*-acting elements generated a synthetic promoter P_{eAOX1} that responds to ethanol with a 1.3-fold higher expression than that of wild-type P_{AOX1} induced by methanol (Fig. 1C) [42]. To design artificial promoters with variable strengths, Zhu et al. constructed a constitutive transcriptional device library composed of 126 transcriptional devices with expression strengths of 16% to 520% of P_{AOX1}, and an inducible transcriptional device library composed of 162 methanol-inducible transcriptional devices with expression strengths of 30% to 500% of P_{AOX1} by modifying tandem binding sequences, spacers and differentiated input promoters (Fig. 1C) [43]. The core region mutation analysis of P_{AOX1} revealed that the promoter strength was significantly impacted by mutations within the TATA box motif, whereas most mutations in other core regions had only minor effects (Fig. 1D), indicating high tolerance of the P_{AOX1} core promoter to small mutations [44]. Additionally, transcript stability and translation initiation were found to be influenced by the 5′ untranslated region (5′ UTR) [40]. Staley et al. conducted site-directed mutagenesis to either shorten or lengthen the 5′ UTR of P_{AOX1} and observed that both alterations had an effect on translation efficiency. It is worth noting that the 5′ UTR of P_{AOX1} may contain crucial regulatory regions [40,45].

Other inducible promoters have been identified in *P. pastoris*, including P_{PHO89}, which is highly induced under phosphate-limited growth conditions [46], and P_{LRA4} and P_{LRA3}, which can be induced by rhamnose and used as alternatives to methanol-induced promoters [47]. Moreover, Vogl et al. also built a bidirectional promoter library with a cumulative expression range of 79-fold, allowing for extremely high levels of exogenous gene expression [48]. Additionally, there are several repressible promoters, such as P_{MET3} (methionine-repressed), P_{SER1} (serine-repressed), P_{THR1} (threonine-repressed), P_{PIS1} (zinc sulfate-repressed) [49], and P_{THI11} (thiamine-repressed) [50]. These promoters can be used to down-regulate genes that cannot be deleted throughout fermentation.

P_{GAP} is the most commonly used constitutive promoter for protein expression in *P. pastoris*, as it maintains stable cellular function without the use of toxic methanol inducer and is suitable for continuous cultivation [32]. Expression of an alkaline phytase under P_{GAP} enabled eight times higher protein level than using promoter P_{AOX1} [51]. A promoter library of 33 mutants was created through mutagenesis of P_{GAP}, with activities ranging from 0.6% to 19.6 times that of the wild-type promoter (Fig. 1D) [52]. A synthetic promoter library was also created by deleting and replicating putative TFBSs, with expression levels ranging from 0.35 to 3.10 times that of wild-type P_{GAP} (Fig. 1C) [53]. Another constitutive promote P_{GCW14}, has been demonstrated to have higher activity than P_{GAP} under various carbon sources, which may also be used for constitutive gene expression in *P. pastoris* [54].

Terminators also play an important role in gene regulation, influencing mRNA abundance [55] and mRNA stability [56], and post-transcriptional interactions with endogenous *trans*-acting factors [57] in *S. cerevisiae*. In *P. pastoris*, Vogl et al. found no significant effect on eGFP expression, outside of the finding that insertion of the NotI restriction site into the terminator of *AOX1* increased the fluorescence intensity by 37% [30]. Ito et al. constructed a library of terminators by testing 72 terminator sequences, including those from *S. cerevisiae, P. pastoris*, and synthetic terminators, and found a 17-fold tunable range of terminator activities [58].

3. Plasmids

Plasmids are an indispensable tool in genetic engineering and have recently been reviewed for their use in *P. pastoris* [29]. Plasmids are typically preferred over genomic integration because of their high transformation efficiencies, and the use of screening markers can significantly affect plasmid copy numbers [59]. Based on their ability to replicate independently in the host cell, plasmids can be classified as either episomal or integrative. Integrative plasmids can be linearized and integrated into the genome, resulting in single-copy integration [60]. Alternatively, they can be integrated into repetitive genome sequences (delta integration and rDNA cluster integration) using high concentrations of antibiotics, leading to multiple-copy integration. [61–63]. Episomal plasmids replicate independently of the genome using autonomously replicating sequences (ARSs) or centromeres. ARSs generally serve as the origins of DNA replication during mitosis, and constitute the primary origins of plasmid replication in yeast host cells [64]. Although a *P. pastoris*-specific ARS has been identified [65], its stability requires improvement [66]. A heterologous ARS derived from *Kluyveromyces lactis* has been shown to provide the best stability for plasmid replication in *P. pastoris* [67], and is now the most widely used ARS in *P. pastoris*. Additionally, a mitochondrial DNA fragment was discovered to function as a novel ARS in *P. pastoris* [68]. Furthermore, it has recently been found that a stable replicating plasmid can be constructed using the entire chromosome 2 centromeric DNA sequence (Cen2). However, the large size of Cen2 limits its application [66].

A selection marker is another essential component for maintaining plasmids in microbial cells [69]. Two commonly used selection markers are auxotrophic markers and antibiotic resistance genes. Several auxotrophic markers such as *HIS4, ADE1*, and *MET2* require the corresponding genes in the chromosome to be deleted. More recently, *PRO3* has been used as a selection marker in *P. pastoris* by constructing a *PRO3* deletion strain, resulting in a proline auxotroph [70]. Antibiotic resistance markers require no modification other than the introduction of a gene on the plasmid that confers resistance to a given antibiotic [71]. Episomal plasmid-based gene expression is not stable for long-term cultivation and thus not suitable for constructing biosynthetic pathways [72,73]. However, they are suitable for expressing the genetic components of CRISPR gene editing systems since these plasmids can be easily removed after genetic engineering.

4. Genome integration sites

The construction of efficient microbial cell factories requires extensive metabolic rewiring and the expression of multiple genes. Traditional plasmid-based expression systems require different genetic markers and are likely to be unstable due to recombination between plasmids [72,74]. Genome integration is considered a feasible alternative approach for the stable expression of multiple genes. In this case, identifying neutral sites, chromosomal loci that can be disrupted without significant effects on cellular physiology and metabolism, is a prerequisite for stable expression of multiple genes. Several neutral sites have been identified in the model yeast *S. cerevisiae* [74–77] and other non-conventional yeasts [78,79], which greatly facilitates the construction of complex and long biosynthetic pathways. In *P. pastoris*, *AOX1* [80], *GAP* [69], as well as the *HIS4* locus [60], have been used for integrating gene expression cassettes. However, these loci might perturb cellular metabolism, and more integration sites are required to expand genome integration capacity in *P. pastoris*. To address this issue, we screened the genome of *P. pastoris* and identified 46 neutral sites that had no significant impact on cell physiology and metabolism [31]. Liu et al. also screened 10 sites for integration of exogenous genes in *P. pastoris* [81], and Gao et al. further identified 23 potential integration sites in *P. pastoris* [82]. These works greatly expand gene integration sites and facilitate extensive metabolic engineering in *P. pastoris*.

5. Genetic editing systems

5.1. Establishment of genetic editing systems

Early gene editing techniques relied on simultaneous recombination of target genes and selection markers [83], which hindered later genetic modification and industrial applications. To enable multiple rounds of

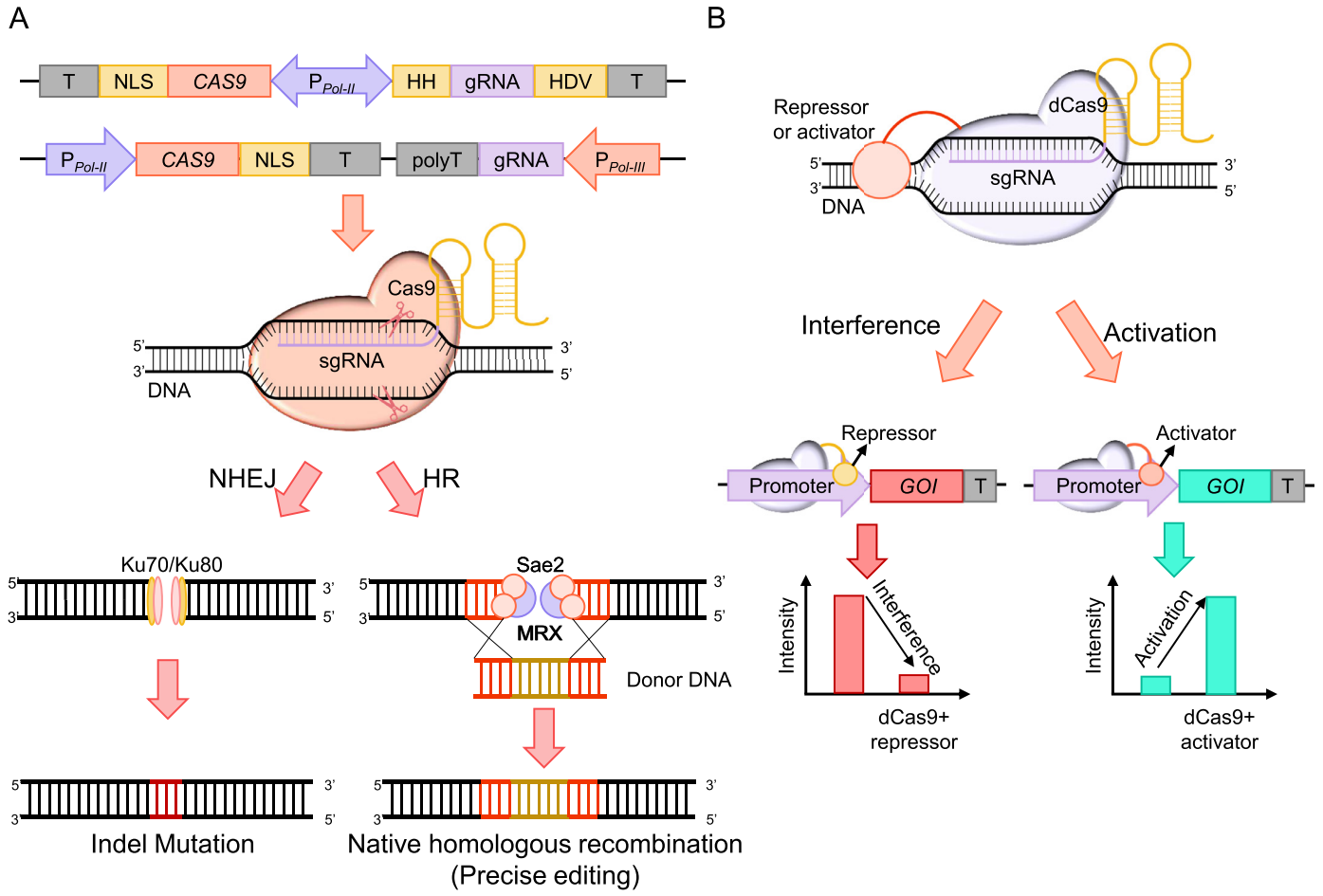

Fig. 2. CRISPR-base genome engineering and gene regulation in *P. pastoris*. (A) The workflow of the CRISPR/Cas9 system: the top portion represents the gRNA design commonly used in *P. pastoris*, the middle portion is the process of DNA cleavage after Cas9-gRNA complex binding, and the bottom portion represents the genome repair process. NHEJ: non-homologous end joining, HR: homologous recombination. MRX: Mre11-Rad50-Xrs2 complex. (B) The working principle of CRISPR/dCas9 system for gene regulation. CRISPRi and CRISPRa are performed through fusion of a repressor or activator protein to deactivated Cas9.

gene editing, several marker-free editing systems have been established in *P. pastoris*. For example, Flp/*FRT* [84,85] and Cre/*loxP* [24,86,87] recombinase-based systems were developed, and the virulence gene *mazF* from *E. coli* was used to construct a set of anti-selection techniques for marker-free genome editing in *P. pastoris* [88]. However, these systems leave unnecessary scars in the genome, which may cause genetic recombination in the host [89].

In recent years, genome editing tools such as zinc-finger nucleases and transcription activator-like effector nucleases have significantly improved the efficiency and accuracy of genetic manipulation [89], but they are not suitable for editing multiple targets simultaneously. In particular, the CRISPR/Cas9 system has revolutionized genome editing [90] (Fig. 2A). Weninger et al. first established CRISPR/Cas9 technology in *P. pastoris* by optimizing the expression of *CAS9* and screening suitable promoters and ribozymes for guide RNA (gRNA) expression [25]. They found that adding an ARS to the donor fragment increased integration rates from 0% to 50% in the wild-type strain, and the CRISPR/Cas9 system enabled 100% seamless gene deletion in the *ku70*Δ strain [91]. ARS screening showed that a panARS-based plasmid for gRNA expression resulted in the highest disruption of the *ADE2* locus [92]. To simplify the construction of gRNA expression plasmid and save primer cost, the *CAS9* gene was integrated into chromosome, and the N6 variable sequence on the gRNA plasmid, which is reverse complementary to the last six base pairs of the hammerhead ribozyme part was fixed [31]. In addition, RNA pol-III type promoters were tested and successfully used for the expression of gRNA without requiring extra ribozyme cleavage [93,94], which enabled a 95% knockout efficiency in *P. pastoris* [95]. The CRISPR/Cas9 system has also been used for multi-loci gene integration [31,81], but the efficiency is relatively low (<40% for three-site integrations) and needs improvement. Outside of the CRISPR/Cas9 system, the CRISPR-Cas12a/Cpf1 system has also been established in *P. pastoris*. Cpf1 produces sticky ends on the target sequence, which are more conducive to host cell repair than blunt ends [96]. Zhang et al. used the CRISPR-Cas12a/Cpf1 system and achieved the deletion of large DNA fragments and integration of multiplexed gene fragments, and the system exhibited a precise and high editing efficiency for single-gene disruption (99%), duplex genome editing (65% to 80%), and triplex genome editing (30%) [97]. Table 2 provides a detailed summary of CRISPR-based genetic engineering systems in *P. pastoris*.

5.2. Improvement of genetic engineering accuracy

The CRISPR/Cas system efficiently generates DNA double-strand breaks (DSBs) through targeted DNA cutting, and DSB repair is essential for precise genetic engineering. Two competing mechanisms for DSB repair in eukaryotes are homologous recombination (HR) and non-homologous end joining (NHEJ) (Fig. 2A). In *P. pastoris*, NHEJ is dominant over HR, resulting in low accuracy of targeted genome engineering, such as inefficient seamless gene deletion and gene integration [98]. Deleting the key NHEJ-related gene *KU70* improved the efficiency of seamless gene deletion to over 90% using 250 bp flanking homologous arms [85]. However, the *ku70*Δ deletion strain became more sensitive

Table 2
Comparison of CRISPR editing systems in *P. pastoris*.

Strain engineering	Nucleases	sgRNA promoter	Genome cutting efficiency[a]	Seamless deletion efficiency	Genome integration efficiency	Minimal HA[b] length	Ref.
/[c]	Cas9	P_{HTB1}	43–95%	2.4%	24% (Single gene)	1000 bp	[25]
*ku70*Δ	Cas9	P_{HTB1}	94%	100%	/	1000 bp	[91]
/	Cas9	P_{SER}	80%	75%	/	250 bp	[92]
*ku70*Δ	Cas9	P_{tRNA1}	93%	/	20% (Three genes)	500 bp	[95]
*ku70*Δ	Cas9	P_{HTB1}	75–98%	/	58–70% (Two genes) 13–32% (Three genes)	1000 bp	[81]
/	Cas9	P_{HTX1}	78%	/	/	/	[106]
Overexpress *RAD52* or *mph1*Δ	Cas9	P_{HTB1}	93%	90%	43–70% (Single gene) 68% (Three genes at one site) 25% (Three genes at three sites)	50 bp	[31]
Overexpress *ScRAD52, ScRAD59* and *ScMRE11*	Cas9	P_{SER}	91%	/	100% (Single gene) 98% (Two genes at two sites) 81% (Three genes at three sites)	40 bp	[100]
Overexpress *RAD52, CAS9-MRE11*	Cas9	P_{HTB1}	92%	87% (Two genes) 17% (Three genes)	92% (Three genes at one site)	1000 bp	[102]
/	Cas12a/Cpf1	P_{SER}	99%	65–80% (Two genes) 30% (Three genes)	/	250 bp	[97]

[a] Efficiency=The number of positive clones/The number of picked clones.
[b] HA: homologous arms.
[c] "/" means not mentioned.

to UV light, and the growth rate decreased by 20%. We also observed that *ku70*Δ resulted in the loss of large genomic fragments when integrating genes at the chromosome terminal [31]. Deletion of the DNA ligase IV gene, *dnl4*Δ, was found to have three times higher HR efficiency than that of the *ku70*Δ strain, and combined knockout showed a 10-fold higher HR efficiency than that of the wild-type strain. In particular, it had a high genome integration efficiency of >70% using 50 bp homology arms [99].

Enhancing the HR procedure has been shown to be a viable alternative to blocking the NHEJ process, as it can improve the accuracy of DSB repair. Our previous work has demonstrated that overexpressing the HR-related gene *RAD52* can increase HR-based DSB repair up to 90% at single-loci and to 25% at triple-loci [31]. Additionally, deleting the endogenous helicase gene *MPH1* can significantly enhance the simultaneous integration of three DNA fragments by 13.5 times [31]. Similarly, Gao et al. showed that overexpression of HR-related genes, such as *RAD52*, *RAD59*, *MRE11*, and *SAE2*, can enable integration of heterologous genes at single-, two-, and three-loci with high efficiencies of 100%, ~98%, and ~81%, respectively [100]. Exonuclease Mre11 can execute short-range resection of DSBs, which makes it conducive to HR repair [101]. Fusion of Mre11 at the C-terminus of Cas9 can improve the positive rates of double-gene deletion (from 76.7% to 86.7%), triple-gene deletion (from 10.8% to 16.7%), as well as the efficiency of simultaneous three-fragment integration (from 66.7% to 91.7%), when combined with *RAD52* overexpression [102]. Engineering HR- or NHEJ-related genes may put stress on cellular fitness, and dynamic regulation of these genes could help to alleviate this stress, making it suitable for constructing robust cell factories.

5.3. Fine-tuning gene expression

Fine-tuning gene expression is a useful strategy for balancing cellular metabolism without completely blocking essential pathways for cellular function. CRISPR-mediated transcriptional regulation approaches involve fusing transcriptional repressors or activators to the nuclease dead Cas9 protein (dCas9) [103], which can achieve CRISPR interference (CRISPRi) [104] or CRISPR activation (CRISPRa) in eukaryotic cells (Fig. 2B) [105]. For example, CRISPRi was used to repress P_{AOX1} activity to 28%-34% after 24 h in *P. pastoris*, which subsequently affected the cell morphology of *P. pastoris* in methanol-induced medium [106]. Similarly, a CRISPRa system was employed to activate the thiamine-repressible promoter, P_{THI11} under repression conditions, resulting in increased riboflavin production [107]. The CRISPR/dCas9 system can perform multiple functions simultaneously, in addition to single gene activation or repression. Recently, a CRISPR based gene regulation system using a single Cas9-activator protein was developed, enabling the simultaneous gene activation, repression, and editing of genes in *P. pastoris* [108]. The development of the programmable high expression yeast platform SynPic-X, achieved through the combination of CRISPR/dCas9 and CRISPR/dCpf1 systems, has been demonstrated as an effective strategy for enabling a programmable expression platform in response to multiple defined signals [109]. Furthermore, biosensor-based metabolic regulation has emerged as another beneficial fine-tuning method for enhancing the production of a target product. Specifically, regulating the content of intermediates can reduce the toxicity of their excessive accumulation. To achieve dynamic regulation of malonyl-CoA in *P. pastoris*, Wen et al. constructed a synthetic malonyl-CoA metabolic oscillator using the FapR/FapO system. This system enabled dynamic regulation of the production and consumption of malonyl-CoA, leading to a significant 260% increase in the titer of malonyl-CoA derived polyketide under the control of this oscillator [110,111].

6. Perspectives and conclusions

P. pastoris is a widely used host for the production of recombinant proteins and small molecules, and efficient genetic tools for metabolic engineering are essential for this purpose. Precise genetic engineering tools can significantly reduce development times and costs compared to time-consuming random mutation. Although CRISPR/Cas systems have significantly improved genetic engineering efficiency in *P. pastoris*, there are areas that require further improvement.

For instance, the efficiency of genome integration in *P. pastoris*, specifically for large DNA fragments, needs further improvement to construct complex biosynthetic pathways [82,100]. Compared to the highly efficient multiplex genome engineering in *S. cerevisiae* [112–114], expressing multiple gRNAs in *P. pastoris* for multiplex genome engineering remains challenging [31,81,100]. This is because current gRNAs are expressed individually and require a cleaving process to obtain functional

gRNAs [31,81,100,102], resulting in low efficiency [115]. However, Cpf1 has the potential process to simplify multiplexed genome editing in *P. pastoris* by processing its own CRISPR RNA and expressing multiple gRNAs in one cassette [116,117]. Additionally, GoldenPiCS, a Golden Gate-derived modular cloning system [118], has been developed as a general-purpose vector for the rapid assembly of multiple genes in *P. pastoris*. Nonetheless, this approach requires the removal of specific restriction enzyme sites within the target gene, promoter, and terminator for proper linearization of the constructed plasmid, thereby increasing the difficulty of plasmid construction [81]. An alternative method, BioBrick, can assemble short strands of synthetic DNA into longer strands through combination of DNA fragments treated with different restriction endonucleases using iterative pairwise assembly without exhausting the unique restriction site [119,120]. BioBrick-compatible parts are relatively facile to design as the upstream prefix sequence and the downstream suffix sequence are the same for each part. Therefore, BioBrick may provide another avenue to link multiple gene segments in *P. pastoris* in the future.

Fine-tuning gene expression is essential for balancing metabolic flux in chemical production. However, the development of such tools in *P. pastoris* lags far behind those in other model microbes [43,109]. Recent advances in new CRISPR/Cas systems, such as type III and VI, have enabled targeting of RNA to regulate gene expression without editing the genomic DNA [121–124]. RNA interference is another tool that can guide the repression of target transcripts [125] and knockdown target genes without genome editing in host cells [126]. Despite these capabilities, these systems have not been established in *P. pastoris*. Thus, further developments of these genetic tools may allow for flexible regulation of gene editing, activation, and repression in *P. pastoris*.

The CRISPR/Cas9 system can also edit a target base in addition to gene knockout, integration, repression, or activation [127]. Single-base editing systems such as cytosine base editor [127], adenine base editor [128], guanine base editor [129], and Prime Editor (PE) [130] have been developed in *S. cerevisiae* [131,132] and *Corynebacterium glutamicum* [133,134]. PE enables not only base replacement, but also accurate insertion and deletion of multiple bases. These editors do not generate DNA DSB and therefore do not activate the DNA double-strand repair machinery, which reduces the risk of introducing indels. None of these editors have been established in *P. pastoris*, making them the focus of future research.

In summary, the development of efficient genetic tools could pave the way for using *P. pastoris* as a microbial cell factory for the production of chemicals, biofuels, and natural products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by supported by the National Key R&D Program of China (2021YFC2103500) and DICP innovation grant (DICP I202111) from Dalian Institute of Chemical Physics, CAS.

References

[1] J.P. Schwarzhans, T. Luttermann, M. Geier, J. Kalinowski, K. Friehs, Towards systems metabolic engineering in *Pichia pastoris*, Biotechnol. Adv. 35 (2017) 681–710.
[2] Z. Yang, Z. Zhang, Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris*: a review, Biotechnol. Adv. 36 (2018) 182–195.
[3] A. Hagman, T. Säll, J. Piškur, Analysis of the yeast short-term Crabtree effect and its origin, FEBS J. 281 (2014) 4805–4814.
[4] D.A. Peña, B. Gasser, J. Zanghellini, M.G. Steiger, D. Mattanovich, Metabolic engineering of *Pichia pastoris*, Metab. Eng. 50 (2018) 2–15.
[5] L. Gao, P. Cai & Y.J. Zhou, Advances in metabolic engineering of methylotrophic yeasts, Sheng Wu Gong Cheng Xue Bao 37 (2021) 966–979.
[6] J.M. Araya-Garay, J.M. Ageitos, J.A. Vallejo, P. Veiga-Crespo, A. Sánchez-Pérez, T.G. Villa, Construction of a novel *Pichia pastoris* strain for production of xanthophylls, AMB Express 2 (2012) 24.
[7] T. Wriessnegger, S. Moser, A. Emmerstorfer-Augustin, E. Leitner, M. Müller, I. Kaluzna, M. Schürmann, D. Mink, H. Pichler, Enhancing cytochrome P450-mediated conversions in *P. pastoris* through *RAD52* over-expression and optimizing the cultivation conditions, Fungal Genet. Biol. 89 (2016) 114–125.
[8] X.B. Liu, M. Liu, X.Y. Tao, Z.X. Zhang, F.Q. Wang, D.Z. Wei, Metabolic engineering of *Pichia pastoris* for the production of dammarenediol-II, J. Biotechnol. 216 (2015) 47–55.
[9] Y. Xue, C. Kong, W. Shen, C. Bai, Y. Ren, X. Zhou, Y. Zhang, M. Cai, Methylotrophic yeast *Pichia pastoris* as a chassis organism for polyketide synthesis via the full citrinin biosynthetic pathway, J. Biotechnol. 242 (2017) 64–72.
[10] Y. Liu, X. Tu, Q. Xu, C. Bai, C. Kong, Q. Liu, J. Yu, Q. Peng, X. Zhou, Y. Zhang, M. Cai, Engineered monoculture and co-culture of methylotrophic yeast for de novo production of monacolin J and lovastatin from methanol, Metab. Eng. 45 (2018) 189–199.
[11] Z. Qian, J. Yu, X. Chen, Y. Kang, Y. Ren, Q. Liu, J. Lu, Q. Zhao, M. Cai, De novo production of plant 4′-deoxyflavones baicalein and oroxylin a from ethanol in crabtree-negative yeast, ACS Synth. Biol. 11 (2022) 1600–1612.
[12] J. Gao, Y. Zuo, F. Xiao, Y. Wang, D. Li, J. Xu, C. Ye, L. Feng, L. Jiang, T. Liu, D. Gao, B. Ma, L. Huang, Z. Xu, J. Lian, Biosynthesis of catharanthine in engineered *Pichia pastoris*, Nat. Synth. 2 (2023) 231–242.
[13] P. Cai, X. Wu, J. Deng, L. Gao, Y. Shen, L. Yao, Y.J. Zhou, Methanol biotransformation toward high-level production of fatty acid derivatives by engineering the industrial yeast *Pichia pastoris*, Proc. Natl. Acad. Sci. U. S. A. 119 (2022) e2201711119.
[14] A. Fina, S. Heux, J. Albiol, P. Ferrer, Combining metabolic engineering and multiplexed screening methods for 3-hydroxyprionic acid production in *Pichia pastoris*, Front. Bioeng. Biotechnol. 10 (2022) 942304.
[15] K. De Schutter, Y.C. Lin, P. Tiels, A. Van Hecke, S. Glinka, J. Weber-Lehmann, P. Rouzé, Y. Van de Peer, N. Callewaert, Genome sequence of the recombinant protein production host *Pichia pastoris*, Nat. Biotechnol. 27 (2009) 561–566.
[16] A. Küberl, J. Schneider, G.G. Thallinger, I. Anderl, D. Wibberg, T. Hajek, S. Jaenicke, K. Brinkrolf, A. Goesmann, R. Szczepanowski, A. Pühler, H. Schwab, A. Glieder, H. Pichler, High-quality genome sequence of *Pichia pastoris* CBS7435, J. Biotechnol. 154 (2011) 312–320.
[17] M. Valli, N.E. Tatto, A. Peymann, C. Gruber, N. Landes, H. Ekker, G.G. Thallinger, D. Mattanovich, B. Gasser, A.B. Graf, Curation of the genome annotation of *Pichia pastoris* (*Komagataella phaffii*) CBS7435 from gene level to protein function, FEMS Yeast Res. 16 (2016) fow051.
[18] L. Sturmberger, T. Chappell, M. Geier, F. Krainer, K.J. Day, U. Vide, S. Trstenjak, A. Schiefer, T. Richardson, L. Soriaga, B. Darnhofer, R. Birner-Gruenberger, B.S. Glick, I. Tolstorukov, J. Cregg, K. Madden, A. Glieder, Refined *Pichia pastoris* reference genome sequence, J. Biotechnol. 235 (2016) 121–131.
[19] K.R. Love, K.A. Shah, C.A. Whittaker, J. Wu, M.C. Bartlett, D. Ma, R.L. Leeson, M. Priest, J. Borowsky, S.K. Young, J.C. Love, Comparative genomics and transcriptomics of *Pichia pastoris*, BMC Genomics 17 (2016) 550.
[20] H. Russmayer, M. Buchetics, C. Gruber, M. Valli, K. Grillitsch, G. Modarres, R. Guerrasio, K. Klavins, S. Neubauer, H. Drexler, M. Steiger, C. Troyer, A. Al Chalabi, G. Krebiehl, D. Sonntag, G. Zellnig, G. Daum, A.B. Graf, F. Altmann, G. Koellensperger, S. Hann, M. Sauer, D. Mattanovich, B. Gasser, Systems-level organization of yeast methylotrophic lifestyle, BMC Biol. 13 (2015) 80.
[21] S.B. Sohn, A.B. Graf, T.Y. Kim, B. Gasser, M. Maurer, P. Ferrer, D. Mattanovich, S.Y. Lee, Genome-scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production, Biotechnol. J. 5 (2010) 705–715.
[22] B.K.S. Chung, S. Selvarasu, A. Camattari, J. Ryu, H. Lee, J. Ahn, H. Lee, D.Y. Lee, Genome-scale metabolic reconstruction and in silico analysis of methylotrophic yeast *Pichia pastoris* for strain improvement, Microb. Cell Fact. 9 (2010) 50.
[23] L. Caspeta, S. Shoaie, R. Agren, I. Nookaew, J. Nielsen, Genome-scale metabolic reconstructions of *Pichia stipitis* and *Pichia pastoris* and in silico evaluation of their potentials, BMC Syst. Biol. 6 (2012) 24.
[24] R. Pan, J. Zhang, W.L. Shen, Z.Q. Tao, S.P. Li, X. Yan, Sequential deletion of *Pichia pastoris* genes by a self-excisable cassette, FEMS Yeast Res. 11 (2011) 292–298.
[25] A. Weninger, A.M. Hatzl, C. Schmid, T. Vogl, A. Glieder, Combinatorial optimization of CRISPR/Cas9 expression enables precision genome engineering in the methylotrophic yeast *Pichia pastoris*, J. Biotechnol. 235 (2016) 139–149.
[26] C. Yan, W. Yu, L. Yao, X. Guo, Y.J. Zhou, J. Gao, Expanding the promoter toolbox for metabolic engineering of methylotrophic yeasts, Appl. Microbiol. Biotechnol. 106 (2022) 3449–3464.
[27] S. He, Z. Zhang, W. Lu, Natural promoters and promoter engineering strategies for metabolic regulation in *Saccharomyces cerevisiae*, J. Ind. Microbiol. Biotechnol. 50 (2023).
[28] O. Cos, R. Ramón, J.L. Montesinos, F. Valero, Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review, Microb. Cell Fact. 5 (2006) 17.
[29] J. Gao, L. Jiang, J. Lian, Development of synthetic biology tools to engineer *Pichia pastoris* as a chassis for the production of natural products, Synth. Syst. Biotechnol. 6 (2021) 110–119.
[30] T. Vogl, L. Sturmberger, T. Kickenweiz, R. Wasmayer, C. Schmid, A.M. Hatzl, M.A. Gerstmann, J. Pitzer, M. Wagner, G.G. Thallinger, M. Geier, A. Glieder, A toolbox of diverse promoters related to methanol utilization: functionally verified parts for heterologous pathway expression in *Pichia pastoris*, ACS Synth. Biol. 5 (2016) 172–186.

[31] P. Cai, X. Duan, X. Wu, L. Gao, M. Ye, Y.J. Zhou, Recombination machinery engineering facilitates metabolic engineering of the industrial yeast *Pichia pastoris*, Nucleic Acids Res 49 (2021) 7791–7805.

[32] A. Turkanoglu Ozcelik, S. Yilmaz, M. Inan, *Pichia pastoris* Promoters, Methods Mol. Biol. 1923 (2019) 97–112.

[33] Y. Xuan, X. Zhou, W. Zhang, X. Zhang, Z. Song, Y. Zhang, An upstream activation sequence controls the expression of *AOX1* gene in *Pichia pastoris*, FEMS Yeast Res. 9 (2009) 1271–1282.

[34] X. Wang, Q. Wang, J. Wang, P. Bai, L. Shi, W. Shen, M. Zhou, X. Zhou, Y. Zhang, M. Cai, Mit1 transcription factor mediates methanol signaling and regulates the Alcohol Oxidase 1 (*AOX1*) Promoter in *Pichia pastoris*, J. Biol. Chem. 291 (2016) 6245–6261.

[35] X. Wang, M. Cai, L. Shi, Q. Wang, J. Zhu, J. Wang, M. Zhou, X. Zhou, Y. Zhang, PpNrg1 is a transcriptional repressor for glucose and glycerol repression of *AOX1* promoter in methylotrophic yeast *Pichia pastoris*, Biotechnol. Lett. 38 (2016) 291–298.

[36] J. Wang, X. Wang, L. Shi, F. Qi, P. Zhang, Y. Zhang, X. Zhou, Z. Song, M. Cai, Methanol-independent protein expression by *AOX1* promoter with trans-acting elements engineering and glucose-glycerol-shift induction in *Pichia pastoris*, Sci. Rep. 7 (2017) 41850.

[37] T. Vogl, L. Sturmberger, P.C. Fauland, P. Hyden, J.E. Fischer, C. Schmid, G.G. Thallinger, M. Geier, A. Glieder, Methanol independent induction in *Pichia pastoris* by simple derepressed overexpression of single transcription factors, Biotechnol. Bioeng. 115 (2018) 1037–1050.

[38] C.H. Chang, H.A. Hsiung, K.L. Hong, C.T. Huang, Enhancing the efficiency of the *Pichia pastoris AOX1* promoter via the synthetic positive feedback circuit of transcription factor Mxr1, BMC Biotechnol. 18 (2018) 81.

[39] F.S. Hartner, C. Ruth, D. Langenegger, S.N. Johnson, P. Hyka, G.P. Lin-Cereghino, J. Lin-Cereghino, K. Kovar, J.M. Cregg, A. Glieder, Promoter library designed for fine-tuned gene expression in *Pichia pastoris*, Nucleic Acids Res. 36 (2008) e76.

[40] T. Vogl, C. Ruth, J. Pitzer, T. Kickenweiz, A. Glieder, Synthetic core promoters for *Pichia pastoris*, ACS Synth. Biol. 3 (2014) 188–191.

[41] R.M. Portela, T. Vogl, C. Kniely, J.E. Fischer, R. Oliveira, A. Glieder, Synthetic core promoters as universal parts for fine-tuning expression in different yeast species, ACS Synth. Biol. 6 (2017) 471–484.

[42] B.G. Ergun, I. Demir, T.H. Ozdamar, B. Gasser, D. Mattanovich, P. Calik, Engineered deregulation of expression in yeast with designed hybrid-promoter architectures in coordination with discovered master regulator transcription factor, Adv. Biosyst. 4 (2020) e1900172.

[43] Q. Zhu, Q. Liu, C. Yao, Y. Zhang, M. Cai, Yeast transcriptional device libraries enable precise synthesis of value-added chemicals from methanol, Nucleic Acids Res. (2022) 10187–10199.

[44] R.M.C. Portela, T. Vogl, K. Ebner, R. Oliveira, A. Glieder, *Pichia pastoris* Alcohol Oxidase 1 (*AOX1*) core promoter engineering by high resolution systematic mutagenesis, Biotechnol. J. 13 (2018) e1700340.

[45] C.A. Staley, A. Huang, M. Nattestad, K.T. Oshiro, L.E. Ray, T. Mulye, Z.H. Li, T. Le, J.J. Stephens, S.R. Gomez, A.D. Moy, J.C. Nguyen, A.H. Franz, J. Lin-Cereghino, G.P. Lin-Cereghino, Analysis of the 5′ untranslated region (5′UTR) of the alcohol oxidase 1 (*AOX1*) gene in recombinant protein expression in *Pichia pastoris*, Gene 496 (2012) 118–127.

[46] J. Ahn, J. Hong, M. Park, H. Lee, E. Lee, C. Kim, J. Lee, E.-s. Choi, J.-k. Jung, H. Lee, Phosphate-responsive promoter of a *Pichia pastoris* sodium phosphate symporter, Appl. Environ. Microbiol. 75 (2009) 3528–3534.

[47] B. Liu, Y. Zhang, X. Zhang, C. Yan, Y. Zhang, X. Xu, W. Zhang, Discovery of a rhamnose utilization pathway and rhamnose-inducible promoters in *Pichia pastoris*, Sci. Rep. 6 (2016) 27352.

[48] T. Vogl, T. Kickenweiz, J. Pitzer, L. Sturmberger, A. Weninger, B.W. Biggs, E.M. Kohler, A. Baumschlager, J.E. Fischer, P. Hyden, M. Wagner, M. Baumann, N. Borth, M. Geier, P.K. Ajikumar, A. Glieder, Engineered bidirectional promoters enable rapid multi-gene co-expression optimization, Nat. Commun. 9 (2018) 3589.

[49] M. Delic, D. Mattanovich, B. Gasser, Repressible promoters – A novel tool to generate conditional mutants in *Pichia pastoris*, Microb. Cell Fact. 12 (2013) 6.

[50] N. Landes, B. Gasser, K. Vorauer-Uhl, G. Lhota, D. Mattanovich, M. Maurer, The vitamin-sensitive promoter P_{THI11} enables pre-defined autonomous induction of recombinant protein production in *Pichia pastoris*, Biotechnol. Bioeng. 113 (2016) 2633–2643.

[51] M. Yang, S. Teymorian, P. Olivares, P.P.N. Murthy, Extracellular expression of alkaline phytase in *Pichia pastoris*: influence of signal peptides, promoters and growth medium, Biotechnol. Rep. 6 (2015) 112–118.

[52] X. Qin, J. Qian, G. Yao, Y. Zhuang, S. Zhang, J. Chu, *GAP* promoter library for fine–tuning of gene expression in *Pichia pastoris*, Appl. Environ. Microbiol. 77 (2011) 3600–3608.

[53] O. Ata, R. Prielhofer, B. Gasser, D. Mattanovich, P. Calik, Transcriptional engineering of the glyceraldehyde-3-phosphate dehydrogenase promoter for improved heterologous protein production in *Pichia pastoris*, Biotechnol. Bioeng. 114 (2017) 2319–2327.

[54] S. Liang, C. Zou, Y. Lin, X. Zhang, Y. Ye, Identification and characterization of P_{GCW14}: a novel, strong constitutive promoter of *Pichia pastoris*, Biotechnol. Lett. 35 (2013) 1865–1871.

[55] M. Yamanishi, Y. Ito, R. Kintaka, C. Imamura, S. Katahira, A. Ikeuchi, H. Moriya, T. Matsuyama, A genome-wide activity assessment of terminator regions in *Saccharomyces cerevisiae* provides a "Terminatome" toolbox, ACS Synth. Biol. 2 (2013) 337–347.

[56] K.A. Curran, A.S. Karim, A. Gupta, H.S. Alper, Use of expression-enhancing terminators in *Saccharomyces cerevisiae* to increase mRNA half-life and improve gene expression control for metabolic engineering applications, Metab. Eng. 19 (2013) 88–97.

[57] Y. Ito, T. Kitagawa, M. Yamanishi, S. Katahira, S. Izawa, K. Irie, M. Furutani-Seiki, T. Matsuyama, Enhancement of protein production via the strong *DIT1* terminator and two RNA-binding proteins in *Saccharomyces cerevisiae*, Sci. Rep. 6 (2016) 36997.

[58] Y. Ito, G. Terai, M. Ishigami, N. Hashiba, Y. Nakamura, T. Bamba, R. Kumokita, T. Hasunuma, K. Asai, J. Ishii, A. Kondo, Exchange of endogenous and heterogeneous yeast terminators in *Pichia pastoris* to tune mRNA stability and gene expression, Nucleic Acids Res. (2020) 13000–13012.

[59] A.S. Karim, K.A. Curran, H.S. Alper, Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications, FEMS Yeast Res. 13 (2013) 107–116.

[60] M.A. Yoshimasu, J.K. Ahn, T. Tanaka, R.Y. Yada, Soluble expression and purification of porcine pepsinogen from *Pichia pastoris*, Protein Expression Purif. 25 (2002) 229–236.

[61] Y. Wang, J. Wang, F. Leng, J. Ma, A. Bagadi, Expression of *Aspergillus niger* glucose oxidase in *Pichia pastoris* and its antimicrobial activity against *Agrobacterium* and *Escherichia coli*, PeerJ 8 (2020) e9010.

[62] R. Yamada, K. Ogura, Y. Kimoto, H. Ogino, Toward the construction of a technology platform for chemicals production from methanol: D-lactic acid production from methanol by an engineered yeast *Pichia pastoris*, World J. Microbiol. Biotechnol. 35 (2019) 37.

[63] K. Malcı, L.E. Walls, L. Rios-Solis, Multiplex genome engineering methods for yeast cell factory development, Front. Bioeng. Biotechnol. 8 (2020) 589468.

[64] L. Bernauer, A. Radkohl, L.G.K. Lehmayer, A. Emmerstorfer-Augustin, *Komagataella phaffii* as emerging model organism in fundamental research, Front. Microbiol. 11 (2020) 607028.

[65] J.M. Cregg, K.J. Barringer, A.Y. Hessler, K.R. Madden, *Pichia pastoris* as a host system for transformations, Mol. Cell. Biol. 5 (1985) 3376–3385.

[66] Y. Nakamura, T. Nishi, R. Noguchi, Y. Ito, T. Watanabe, T. Nishiyama, S. Aikawa, T. Hasunuma, J. Ishii, Y. Okubo, A. Kondo, A. Stable, Autonomously replicating plasmid vector containing *Pichia pastoris* centromeric DNA, Appl. Environ. Microbiol. 84 (2018) e02882 -02817.

[67] A. Camattari, A. Goh, L.Y. Yip, A.H. Tan, S.W. Ng, A. Tran, G. Liu, I. Liachko, M.J. Dunham, G. Rancati, Characterization of a panARS-based episomal vector in the methylotrophic yeast *Pichia pastoris* for recombinant protein production and synthetic biology applications, Microb, Cell. Fact. 15 (2016) 139.

[68] J.P. Schwarzhans, T. Luttermann, D. Wibberg, A. Winkler, W. Hubner, T. Huser, J. Kalinowski, K. Friehs, A mitochondrial autonomously replicating sequence from *Pichia pastoris* for uniform high level recombinant protein production, Front. Microbiol. 8 (2017) 780.

[69] D. Mattanovich, M. Sauer & B. Gasser. Industrial Microorganisms: *pichia pastoris*, in: C. Wittmann and J.C. Liao (Eds.), Industrial Biotechnology, 2017, pp. 687–714.

[70] M. Ahmad, C.M. Winkler, M. Kolmbauer, H. Pichler, H. Schwab, A. Emmerstorfer-Augustin, *Pichia pastoris* protease-deficient and auxotrophic strains generated by a novel, user-friendly vector toolbox for gene deletion, Yeast (2019) 557–570.

[71] Z. Fatma, J.C. Schultz, H. Zhao, Recent advances in domesticating non-model microorganisms, Biotechnol. Prog. 36 (2020) e3008.

[72] W. Yu, X. Cao, J. Gao, Y.J. Zhou, Overproduction of 3-hydroxypropionate in a super yeast chassis, Bioresour. Technol. 361 (2022) 127690.

[73] P. Cai, Y. Li, X. Zhai, L. Yao, X. Ma, L. Jia, Y.J. Zhou, Microbial synthesis of long-chain α-alkenes from methanol by engineering *Pichia pastoris*, Bioresour. Bioprocess. 9 (2022) 58.

[74] M.D. Mikkelsen, L.D. Buron, B. Salomonsen, C.E. Olsen, B.G. Hansen, U.H. Mortensen, B.A. Halkier, Microbial production of indolylglucosinolate through engineering of a multi-gene pathway in a versatile yeast expression platform, Metab. Eng. 14 (2012) 104–111.

[75] D. Bai Flagfeldt, V. Siewers, L. Huang, J. Nielsen, Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*, Yeast 26 (2009) 545–551.

[76] M. Babaei, L. Sartori, A. Karpukhin, D. Abashkin, E. Matrosova, I. Borodina, Expansion of EasyClone-MarkerFree toolkit for *Saccharomyces cerevisiae* genome with new integration sites, FEMS Yeast Res. 21 (2021) foab027.

[77] S. Kong, W. Yu, N. Gao, X. Zhai, Y.J. Zhou, Expanding the neutral sites for integrated gene expression in *Saccharomyces cerevisiae*, FEMS Microbiol. Lett. 369 (2022) fnac081.

[78] W. Yu, J. Gao, X. Zhai, Y.J. Zhou, Screening neutral sites for metabolic engineering of methylotrophic yeast *Ogataea polymorpha*, Synth. Syst. Biotechnol. 6 (2021) 63–68.

[79] X. Liu, Z. Cui, T. Su, X. Lu, J. Hou, Q. Qi, Identification of genome integration sites for developing a CRISPR-based gene expression toolkit in *Yarrowia lipolytica*, Microb. Biotechnol. 15 (2022) 2223–2234.

[80] T. Papakonstantinou, S. Harris, M.T.W. Hearn, Expression of GFP using *Pichia pastoris* vectors with zeocin or G-418 sulphate as the primary selectable marker, Yeast 26 (2009) 311–321.

[81] Q. Liu, X. Shi, L. Song, H. Liu, X. Zhou, Q. Wang, Y. Zhang, M. Cai, CRISPR–Cas9-mediated genomic multiloci integration in *Pichia pastoris*, Microb. Cell. Fact. 18 (2019) 144.

[82] J. Gao, J. Xu, Y. Zuo, C. Ye, L. Jiang, L. Feng, L. Huang, Z. Xu, J. Lian, Synthetic biology toolkit for marker-less integration of multigene pathways into *Pichia pastoris* via CRISPR/Cas9, ACS Synth. Biol. 11 (2022) 623–633.

[83] J.H. Nett, T.U. Gerngross, Cloning and disruption of the *PpURA5* gene and construction of a set of integration vectors for the stable genetic modification of *Pichia pastoris*, Yeast 20 (2003) 1279–1290.

[84] J.M. Cregg, K.R. Madden, Use of site-specific recombination to regenerate selectable markers, Mol. Gen. Genet. 219 (1989) 320–323.

[85] L. Naatsaari, B. Mistlberger, C. Ruth, T. Hajek, F.S. Hartner, A. Glieder, Deletion of the *Pichia pastoris KU70* homologue facilitates platform strain generation for gene expression and synthetic biology, PLoS ONE 7 (2012) e39720.
[86] H. Marx, D. Mattanovich, M. Sauer, Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris*, Microb. Cell Fact. 7 (2008) 23.
[87] D. Li, B. Zhang, S. Li, J. Zhou, H. Cao, Y. Huang, Z. Cui, A novel vector for construction of markerless multicopy overexpression Transformants in *Pichia pastoris*, Front. Microbiol. 8 (2017) 1698.
[88] J. Yang, W. Jiang, S. Yang, *mazF* as a counter-selectable marker for unmarked genetic modification of *Pichia pastoris*, FEMS Yeast Res. 9 (2009) 600–609.
[89] P. Cai, J. Gao, Y.J. Zhou, CRISPR-mediated genome editing in non-conventional yeasts for biotechnological applications, Microb. Cell. Fact. 18 (2019) 63.
[90] Patrick D. Hsu, Eric S. Lander, &.F. Zhang, Development and applications of CRISPR-Cas9 for genome engineering, Cell 157 (2014) 1262–1278.
[91] A. Weninger, J.E. Fischer, H. Raschmanova, C. Kniely, T. Vogl, A. Glieder, Expanding the CRISPR/Cas9 toolkit for *Pichia pastoris* with efficient donor integration and alternative resistance markers, J. Cell. Biochem. 119 (2018) 3183–3198.
[92] Y. Gu, J. Gao, M. Cao, C. Dong, J. Lian, L. Huang, J. Cai, Z. Xu, Construction of a series of episomal plasmids and their application in the development of an efficient CRISPR/Cas9 system in *Pichia pastoris*, World J. Microbiol. Biotechnol. 35 (2019) 79.
[93] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems, Science 339 (2013) 819–823.
[94] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, G.M. Church, RNA-guided human genome engineering via Cas9, Science 339 (2013) 823–826.
[95] N.C. Dalvie, J. Leal, C.A. Whittaker, Y. Yang, J.R. Brady, K.R. Love, J.C. Love, Host-informed expression of CRISPR guide RNA for genomic engineering in *Komagataella phaffii*, ACS Synth. Biol. 9 (2020) 26–35.
[96] B. Zetsche, Jonathan S. Gootenberg, Omar O. Abudayyeh, Ian M. Slaymaker, Kira S. Makarova, P. Essletzbichler, Sara E. Volz, J. Joung, J. van der Oost, A. Regev, Eugene V. Koonin, &.F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (2015) 759–771.
[97] X. Zhang, S. Gu, X. Zheng, S. Peng, Y. Li, Y. Lin, S. Liang, A novel and efficient genome editing tool assisted by CRISPR-Cas12a/Cpf1 for *Pichia pastoris*, ACS Synth. Biol. 10 (2021) 2927–2937.
[98] U. Klinner, B. Schäfer, Genetic aspects of targeted insertion mutagenesis in yeasts, FEMS Microbiol. Rev. 28 (2004) 201–223.
[99] Y. Ito, T. Watanabe, S. Aikawa, T. Nishi, T. Nishiyama, Y. Nakamura, T. Hasunuma, Y. Okubo, J. Ishii, A. Kondo, Deletion of DNA ligase IV homolog confers higher gene targeting efficiency on homologous recombination in *Komagataella phaffii*, FEMS Yeast Res. 18 (2018) foy074.
[100] J. Gao, C. Ye, J. Cheng, L. Jiang, X. Yuan, J. Lian, Enhancing homologous recombination efficiency in *Pichia pastoris* for multiplex genome integration using short homology arms, ACS Synth. Biol. 11 (2022) 547–553.
[101] E. Casari, C. Rinaldi, A. Marsella, M. Gnugnoli, C.V. Colombo, D. Bonetti, M.P. Longhese, Processing of DNA double-strand breaks by the MRX complex in a chromatin context, Front. Mol. Biosci. 6 (2019) 43.
[102] K. Zhang, X. Duan, P. Cai, L. Gao, X. Wu, L. Yao, Y.J. Zhou, Fusing an exonuclease with Cas9 enhances homologous recombination in *Pichia pastoris*, Microb. Cell Fact. 21 (2022) 182.
[103] X. Xu, L.S. Qi, A CRISPR–dCas toolbox for genetic engineering and synthetic biology, J. Mol. Biol. 431 (2019) 34–47.
[104] Lei S. Qi, Matthew H. Larson, Luke A. Gilbert, Jennifer A. Doudna, Jonathan S. Weissman, Adam P. Arkin, Wendell A. Lim, Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression, Cell 152 (2013) 1173–1183.
[105] Luke A. Gilbert, Matthew H. Larson, L. Morsut, Z. Liu, Gloria A. Brar, Sandra E. Torres, N. Stern-Ginossar, O. Brandman, Evan H. Whitehead, Jennifer A. Doudna, Wendell A. Lim, Jonathan S. Weissman, Lei S. Qi, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes, Cell 154 (2013) 442–451.
[106] Y. Yang, G. Liu, X. Chen, M. Liu, C. Zhan, X. Liu, Z. Bai, High efficiency CRISPR/Cas9 genome editing system with an eliminable episomal sgRNA plasmid in *Pichia pastoris*, Enzyme Microb. Technol. 138 (2020) 109556.
[107] M. Baumschabl, R. Prielhofer, D. Mattanovich, M.G. Steiger, Fine-tuning of transcription in *Pichia pastoris* using dCas9 and RNA Scaffolds, ACS Synth. Biol. 9 (2020) 3202–3209.
[108] X. Liao, L. Li, A. Jameel, X.H. Xing, C. Zhang, A versatile toolbox for CRISPR-based genome engineering in *Pichia pastoris*, Appl. Microbiol. Biotechnol. 105 (2021) 9211–9218.
[109] Q. Liu, L. Song, Q. Peng, Q. Zhu, X. Shi, M. Xu, Q. Wang, Y. Zhang, M. Cai, A programmable high-expression yeast platform responsive to user-defined signals, Sci. Adv. 8 (2022) eabl5166.
[110] J. Wen, L. Tian, M. Xu, X. Zhou, Y. Zhang, M. Cai, A synthetic malonyl-CoA metabolic oscillator in *Komagataella phaffii*, ACS Synth. Biol. 9 (2020) 1059–1068.
[111] J. Wen, L. Tian, Q. Liu, Y. Zhang, M. Cai, Engineered dynamic distribution of malonyl-CoA flux for improving polyketide biosynthesis in *Komagataella phaffii*, J. Biotechnol. 320 (2020) 80–85.
[112] Z.X. Zhang, L.R. Wang, Y.S. Xu, W.T. Jiang, T.Q. Shi, X.M. Sun, H. Huang, Recent advances in the application of multiplex genome editing in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 105 (2021) 3873–3882.
[113] M. Qi, B. Zhang, L. Jiang, S. Xu, C. Dong, Y.L. Du, Z. Zhou, L. Huang, Z. Xu, J. Lian, PCR & Go: a pre-installed expression chassis for facile integration of multi-gene biosynthetic pathways, Front. Bioeng. Biotechnol. 8 (2021) 613771.
[114] M. Wijsman, M.A. Świat, W.L. Marques, J.K. Hettinga, M. van den Broek, P. d. l. Torre Cortés, R. Mans, J.T. Pronk, J.M. Daran, P. Daran-Lapujade, A toolkit for rapid CRISPR-SpCas9 assisted construction of hexose-transport-deficient *Saccharomyces cerevisiae* strains, FEMS Yeast Res. 19 (2018) foy107.
[115] J.C. Utomo, C.L. Hodgins, & D.-K. Ro, Multiplex genome editing in yeast by CRISPR/Cas9 – a potent and agile tool to reconstruct complex metabolic pathways, Front, Plant Sci 12 (2021) 719148.
[116] B. Paul, G. Montoya, CRISPR-Cas12a: functional overview and applications, Biomed. J. 43 (2020) 8–17.
[117] B. Zetsche, M. Heidenreich, P. Mohanraju, I. Fedorova, J. Kneppers, E.M. DeGennaro, N. Winblad, S.R. Choudhury, O.O. Abudayyeh, J.S. Gootenberg, W.Y. Wu, D.A. Scott, K. Severinov, J. van der Oost, F. Zhang, Multiplex gene editing by CRISPR–Cpf1 using a single crRNA array, Nat. Biotechnol. 35 (2017) 31–34.
[118] R. Prielhofer, J.J. Barrero, S. Steuer, T. Gassler, R. Zahrl, K. Baumann, M. Sauer, D. Mattanovich, B. Gasser, H. Marx, GoldenPiCS: a Golden Gate-derived modular cloning system for applied synthetic biology in the yeast *Pichia pastoris*, BMC Syst. Biol. 11 (2017) 123.
[119] T. Knight, Idempotent Vector Design For Standard Assembly of Biobricks, MIT Synthetic Biology Working Group, 2003.
[120] I. Matsumura, Golden Gate assembly of BioBrick-compliant parts using Type II restriction endonucleases, BioTechniques 72 (2022) 185–193.
[121] L. Yang, L.L. Chen, Enhancing the RNA engineering toolkit, Science 358 (2017) 996–997.
[122] J. Zhang, S. Graham, A. Tello, H. Liu, M.F. White, Multiple nucleic acid cleavage modes in divergent type III CRISPR systems, Nucleic Acids Res. 44 (2016) 1789–1799.
[123] O.O. Abudayyeh, J.S. Gootenberg, S. Konermann, J. Joung, I.M. Slaymaker, D.B.T. Cox, S. Shmakov, K.S. Makarova, E. Semenova, L. Minakhin, K. Severinov, A. Regev, E.S. Lander, E.V. Koonin, F. Zhang, C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector, Science 353 (2016) aaf5573.
[124] A. East-Seletsky, M.R. O'Connell, S.C. Knight, D. Burstein, J.H.D. Cate, R. Tjian, J.A. Doudna, Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection, Nature 538 (2016) 270–273.
[125] T.A. Farazi, S.A. Juranek, T. Tuschl, The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members, Development 135 (2008) 1201–1214.
[126] I.A. Drinnenberg, D.E. Weinberg, K.T. Xie, J.P. Mower, K.H. Wolfe, G.R. Fink, D.P. Bartel, RNAi in Budding Yeast, Science 326 (2009) 544–550.
[127] A.C. Komor, Y.B. Kim, M.S. Packer, J.A. Zuris, D.R. Liu, Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage, Nature 533 (2016) 420–424.
[128] N.M. Gaudelli, A.C. Komor, H.A. Rees, M.S. Packer, A.H. Badran, D.I. Bryson, D.R. Liu, Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage, Nature 551 (2017) 464–471.
[129] D. Zhao, J. Li, S. Li, X. Xin, M. Hu, M.A. Price, S.J. Rosser, C. Bi, X. Zhang, Glycosylase base editors enable C-to-A and C-to-G base changes, Nat. Biotechnol. 39 (2021) 35–40.
[130] A.V. Anzalone, P.B. Randolph, J.R. Davis, A.A. Sousa, L.W. Koblan, J.M. Levy, P.J. Chen, C. Wilson, G.A. Newby, A. Raguram, D.R. Liu, Search-and-replace genome editing without double-strand breaks or donor DNA, Nature 576 (2019) 149–157.
[131] Y. Liu, Y. Lin, Y. Guo, F. Wu, Y. Zhang, X. Qi, Z. Wang, Q. Wang, Stress tolerance enhancement via *SPT15* base editing in *Saccharomyces cerevisiae*, Biotechnol. Biofuels 14 (2021) 155.
[132] J. Tan, F. Zhang, D. Karcher, R. Bock, Expanding the genome-targeting scope and the site selectivity of high-precision base editors, Nat. Commun. 11 (2020) 629.
[133] Y. Liu, R. Wang, J. Liu, H. Lu, H. Li, Y. Wang, X. Ni, J. Li, Y. Guo, H. Ma, X. Liao, M. Wang, Base editor enables rational genome-scale functional screening for enhanced industrial phenotypes in *Corynebacterium glutamicum*, Sci. Adv. 8 (2022) eabq2157.
[134] Y.B. Heo, G.H. Hwang, S.W. Kang, S. Bae, H.M. Woo, High-fidelity cytosine base editing in a GC-Rich *Corynebacterium glutamicum* with reduced DNA off-target editing effects, Microbiol. Spectr. 10 (2022) e03760 -03722.
[135] R. Prielhofer, M. Maurer, J. Klein, J. Wenger, C. Kiziak, B. Gasser, D. Mattanovich, Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*, Microb. Cell Fact. 12 (2013) 5.
[136] G. Stadlmayr, A. Mecklenbräuker, M. Rothmüller, M. Maurer, M. Sauer, D. Mattanovich, B. Gasser, Identification and characterisation of novel *Pichia pastoris* promoters for heterologous protein production, J. Biotechnol. 150 (2010) 519–529.
[137] A. Massahi, P. Çalık, Naturally occurring novel promoters around pyruvate branch–point for recombinant protein production in *Pichia pastoris* (*Komagataella phaffii*): pyruvate decarboxylase- and pyruvate kinase- promoters, Biochem. Eng. J. 138 (2018) 111–120.
[138] M. Karaoglan, F.E. Karaoglan, M. Inan, Comparison of *ADH3* promoter with commonly used promoters for recombinant protein production in *Pichia pastoris*, Protein Expression Purif. 121 (2016) 112–117.
[139] N. Xu, J. Zhu, Q. Zhu, Y. Xing, M. Cai, T. Jiang, M. Zhou, Y. Zhang, Identification and characterization of novel promoters for recombinant protein production in yeast *Pichia pastoris*, Yeast 35 (2018) 379–385.