Journal of Industrial Microbiology and Biotechnology, 2023, 50, kuac029
https://doi.org/10.1093/jimb/kuac029
Advance access publication date: 12 January 2023
Metabolic Engineering and Synthetic Biology – Review

Natural promoters and promoter engineering strategies for metabolic regulation in *Saccharomyces cerevisiae*

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Abstract: *Sharomyces cerevisiae* is currently one of the most important foreign gene expression systems. *S. cerevisiae* is an excellent host for high-value metabolite cell factories due to its advantages of simplicity, safety, and nontoxicity. A promoter, as one of the basic elements of gene transcription, plays an important role in regulating gene expression and optimizing metabolic pathways. Promoters control the direction and intensity of transcription, and the application of promoters with different intensities and performances will largely determine the effect of gene expression and ultimately affect the experimental results. Due to its significant role, there have been many studies on promoters for decades. While some studies have explored and analyzed new promoters with different functions, more studies have focused on artificially modifying promoters to meet their own scientific needs. Thus, this article reviews current research on promoter engineering techniques and related natural promoters in *S. cerevisiae*. First, we introduce the basic structure of promoters and the classification of natural promoters. Then, the classification of various promoter strategies is reviewed. Finally, by grouping related articles together using various strategies, this review anticipates the future development direction of promoter engineering.

Keywords: Promoters, *Saccharomyces cerevisiae*, Gene expression, Biosensor

Introduction

The first eukaryotic organism to complete whole genome sequencing (Goffeau et al., 1996) was *S. cerevisiae*. There are numerous advantages to using *S. cerevisiae* as a host, including a clear genetic background, simple operation, safety and nontoxicity, high expression levels of foreign genes, proper enzyme activity, and so on (Borodina & Nielsen, 2014). As a result of its excellent results in various types of research, *S. cerevisiae* has become the most important foreign gene expression system to date (Borodina & Nielsen, 2014). In addition, because *S. cerevisiae* can provide a similar physiological environment for the functional expression of a wide range of heterologous enzymes, it has emerged as a popular microbial cell factory for the biosynthesis of high-value metabolites (Dai et al., 2015).

The transcription unit in *S. cerevisiae*, as we all know, is made up of a promoter, a coding sequence, and a terminator. Promoters, as a fundamental component of transcription, can control the opening and intensity of transcription and are critical factors in finely regulating gene expression at various levels (Redden et al., 2015; Struhl, 1995). Therefore, many studies have been conducted on promoters for decades, and many databases have been obtained on *S. cerevisiae* promoters. These databases which integrate and display key structural information such as the transcription factor binding site (TFBS), TATA box, and transcription start site (TSS) in the *S. cerevisiae* promoter, make it easier for researchers to conduct promoter research (Chang et al., 2011; Matys et al., 2006; Portales-Casamar et al., 2010; Zhu & Zhang, 1999). Initially, applications of promoters in the genetic manipulation of *S. cerevisiae* were mainly derived from the excavation of endogenous sequences (Redden et al., 2015). In recent years, rational designs of *S. cerevisiae* promoters allowed people to obtain a larger number of promoters with a wider range of initiation strengths and finer transcriptional regulation (L. Q. Jin et al., 2019). Another way to create a biological component that dynamically controls gene expression is to combine a promoter with other biological components. Designing and utilizing promoter elements to balance and coordinate gene expression in organisms has gained in significance with the development of synthetic biology (David et al., 2016; Peng et al., 2022). With the rapid development of computer science, research that combines computers and biology has also improved the possibility of promoter engineering (Vaishnav et al., 2022).

This review introduces the main structure and classification of *S. cerevisiae* promoters, as well as various strategies for promoter modification and optimization, based on the importance of promoters in metabolic engineering and synthetic biology. Until now, studying various strategies has resulted in the development of many powerful promoters; however, more methods are required to further expand the *S. cerevisiae* promoter library and obtain superior constitutive or inducible engineering promoters. We discuss the possibility of combining different promoter engineering strategies here, based on the mature single strategy. The combination of different strategies may lead to more powerful engineering promoters with excellent performance.

Received: May 19, 2022. **Accepted:** December 19, 2022.

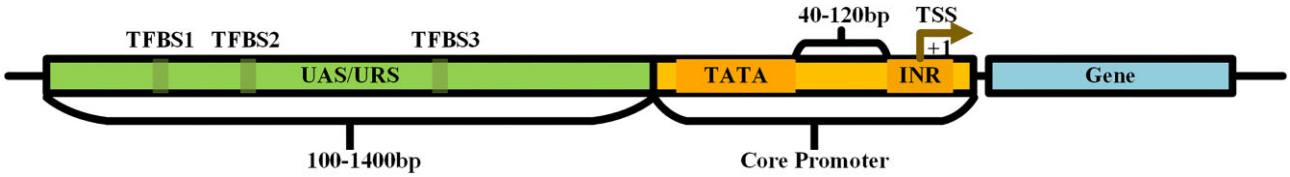

Fig. 1. Structure of *Saccharomyces cerevisiae* promoter. UAS/URS are shown in light green, which represent upstream activating/repressing sequences in the promoter; TFBSs are shown in dark green, which represent transcription factor binding sites; Core promoter is shown in light orange; TATA-box is shown in dark orange, which is the binding site of TATA-binding protein; INR is shown in dark orange, which represents the initiator; TSS is shown on the brown arrow, which represents the transcription start site.

Natural Promoters

The Structure of *S. cerevisiae* Promoters

Promoters are the basic elements of gene transcription that regulate the initiation and intensity of transcription. *S. cerevisiae* promoters (Fig. 1) are roughly divided into two parts: the regulatory component, which determines the intensity of transcription, and the transcriptional component. The other component is the core component, which determines the transcription direction and starts site (Hahn & Young, 2011).

S. cerevisiae promoter regulatory components have an upstream activating sequence (UAS) or upstream repressing sequence (URS) (Hampsey, 1998). Regulatory components are roughly located 100–1400 bp upstream of the core component of promoters (Feng & Marchisio, 2021b), containing one or more TFBSs, which activate or inhibit transcription by binding to specific transcription factors (TFs) (Zhu et al., 2009). Regulatory components changing in number and location will affect gene expression level which is guided by the corresponding promoter (Swamy et al., 2009). It has also been shown that UAS (such as UAS_{TEF2}) can act as an insulator for chromosomal regulation of heterologous pathways against position effects in *S. cerevisiae* (Su et al., 2022).

The *S. cerevisiae* promoter's core component is the smallest region required to initiate transcription. Approximately 20% of the *S. cerevisiae* promoter core components contain the TATA box, which is approximately 40–120 bp upstream of the TSS (Struhl, 1987). The position of the TATA box is the binding site of the TATA-binding protein (TBP), and the combination of the TATA box and TATA-binding protein is the first step for RNA polymerase II to initiate transcription. The TATA box and TSS work together to determine the direction of transcriptions (Hubmann et al., 2014; Klein & Struhl, 1994; Struhl, 1995). The sequence around TSS is sometimes called the initiator INR, which plays a prominent role in the initiation of transcription. Although the TATA box can activate transcriptions other than low-level transcription guided by INR, the activation is not limited to the TATA box and is not dependent on the TATA box. Therefore, the effect of INR may be superior for some promoters that do not contain the TATA box (Smale & Baltimore, 1989; Zhang & Dietrich, 2005).

Classification of Natural Promoters

Endogenous promoters

In the process of constructing *S. cerevisiae* cell factories, different natural promoters are often used to regulate the expression of heterologous genes according to different purposes. All those promoters are divided into *S. cerevisiae* endogenous promoters and heterologous promoters. Endogenous promoters are divided into constitutive and inducible promoters based on their functions. The selection of promoters often affects expressions of target genes to a large extent and the yield of products. For example, in a study of lycopene biosynthesis, Bahieldin et al. (2014) used glucose depletion inducible promoter pADH2 to guide lycopene synthesis genes *CrtE*, *CrtB*, and *CrtI* in *S. cerevisiae*, leading to a lycopene output of 3.3 mg/g dry cell weight. In 2019, Li et al. (2019) compared the effects of constitutive and inducible promoters on the synthesis of lycopene in *S. cerevisiae* and found that constitutive promoters used to control pathway expression balance lycopene synthesis and chassis metabolism more effectively, resulting in the lycopene yield of 115.64 mg/L. Recently, Xu et al. (2021) also adopted a constitutive promoter expression strategy to achieve a high-efficiency synthesis of lycopene in *S. cerevisiae*, which provides a basis for further optimizing the synthesis of lycopene. Therefore, it is necessary to clarify the difference between various promoters for metabolic engineering and synthetic biology research.

Constitutive promoters

Constitutive promoters are unaffected by growth environment or stage, and the gene expression levels they guide are always relatively stable, making them widely used in metabolic engineering and synthetic biology. In the past few decades, many endogenous promoters of *S. cerevisiae* have been identified and used. Among them, promoters derived from the glycolytic pathway are most widely used, such as pTDH3 (Holland & Holland, 1980), pPGK1 (Ogden et al., 1986), pADH1 (Hitzeman et al., 1981), and pPDC1 (Kellermann et al., 1986). In addition, cytochrome C isoform promoter pCYC1 (Guarente et al., 1984) and translation elongation factor promoter pTEF1 (Gatignol et al., 1990) are also commonly used. In recent years, researchers have carried out a more systematic identification of promoters in *S. cerevisiae*. Yuan et al. (2017) constructed a genome-wide promoter library of *S. cerevisiae*, used yellow fluorescent protein reporter genes to compare promoter strengths, and used these promoters to drive the expression of xylose-utilizing genes, improving fermentation efficiency. Similarly, Gao et al. (2020) precisely compared the transcriptional and translational strengths of 66 promoters from *S. cerevisiae*. The efficient biosynthesis of (2S)-naringenin from coumaric acid was finally achieved using these promoters to fine-tune gene expression, with the highest titer reaching 1.21 g/L (Gao et al., 2020). Focusing on mitochondria, Dong et al. (2021) identified 20 mitochondria-targeted promoters and used these promoters to achieve increased α-santalene production. Deep learning has been used for promoter sequence identification since the development of machine learning. Recently, a study performed transfer learning on deep residual networks (ResNet). They extracted features from organisms with a large number of promoter data and evaluated four biological promoter data sets including *S. cerevisiae* (Liu et al., 2022).

The strength of constitutive promoters determines the intensity of gene expression. Different genes have different expression requirements. Therefore, it is very important to select the most suitable promoter to guide gene work better. Based on this

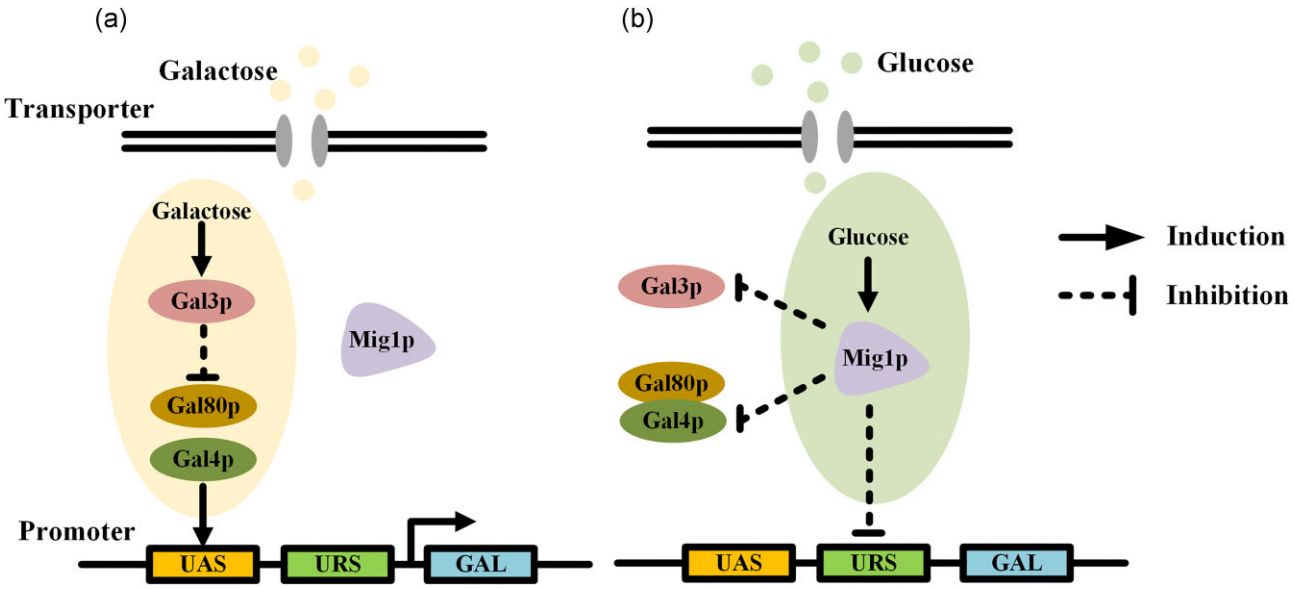

Fig. 2. Regulation mechanism of GAL gene in the presence of galactose or glucose. (a) Expression of the GAL gene in the presence of galactose. Galactose activates *Gal3p*, which prevents *Gal80p* from binding to *Gal4p*, resulting in *Gal4p* activation of GAL gene expression. (b) Inhibition of the GAL gene in the presence of glucose. In the absence of galactose, *Gal80p* and *Gal4p* form a complex to inhibit the activation of GAL gene, and in the presence of glucose, the expression of the transcriptional repressor *Mig1p* is activated to inhibit the expression of GAL gene.

requirement, some studies have constructed different expression vectors and compared the strength of different constitutive promoters in *S. cerevisiae* (Monfort et al., 1999; Mumberg et al., 1995), verifying some powerful natural promoters such as pHXT7 (Jörg et al., 2000) and pTEF1 (Partow et al., 2010; Sun et al., 2012; Zhang et al., 2017). de Paiva et al. (2018) used eGFP as a reporter gene to compare the expression intensity of five promoters (pCYC1, pTEF1, pPGI1, pPGK1, and pADH1) from industrial strains and laboratory strains (S288C). Surprisingly, the researchers found that promoters from different sources had various strengths, even though both strains are *S. cerevisiae*. These findings highlight the significance of carefully selecting heterologous promoters to express target genes. In addition, Ahn et al. (2013) changed the inducible promoter pGAL into a constitutive promoter, so that it could efficiently promote gene expression without galactose induction by removing the GAL80 expression gene. By choosing *lipase B* from *Candida Antarctica* as a reporter, the author compared relative intensities of pGAL10, pADH1, pPDC1, and pPGK in the Δgal80 mutant strain. In this case, the promoter strength of pGAL10 was 0.8 times that of pADH1, 4 times that of pPDC1, and 50 times that of pPGK, proving the feasibility of converting inducible promoters into constitutive promoters for heterologous pathway expression (Ahn et al., 2013).

Inducible promoters

Inducible promoters are those that can greatly increase or decrease expression intensity in response to specific signal stimuli. Therefore, by controlling the content of these specific signals in a culture medium, it is possible to control the expression of specific proteins or to separate cell growth and product synthesis stages, avoiding damage to heterologous toxic proteins. However, this does not mean that inducible promoter is necessarily stronger than constitutive promoters. Chen et al. (2022), for example, used constitutive promoter pPGK1 to replace natural promoters of *PRP6*, *IPL1*, and *RTC1* whose expressions are responsive to zinc sulfate, and the engineered yeast strain turns out to improve the titer of ethanol (Chen et al., 2022). This suggests that the rational use of constitutive and inducible promoters is a better choice.

In general, the inducer, basic activity, and induction factor of promoters must all be considered when selecting inducible promoters. Here are some common inducible promoters.

The first is galactose-induced promoters pGAL1, pGAL2, pGAL3, pGAL7, pGAL10, pGAL80, pMEL1, and so on (Fig. 2). These are the most widely used inducible promoters in *S. cerevisiae*. Their regulation is quite strict, and they are strongly inhibited in the presence of glucose, while their activation strength is greatly increased in the presence of galactose (Adams, 1972; Bassel & Mortimer, 1971). Research on the mechanism of *GAL* gene induction helps to make better use of these promoters (Fig. 2). It is generally believed that the response of the GAL system to galactose signals is related to *GAL4p*, *GAL80p*, and *GAL3p* and is highly sensitive to the relative levels of these three proteins, too. The transcriptional activator *GAL4p* activates the expression of the *GAL* gene by binding to the activation sequence which is 17 bp upstream of the *GAL* gene promoter. In the absence of galactose, the transcription repressor *GAL80p* inhibits the expression of the *GAL* gene by binding to *GAL4p*. When the cell is in the presence of galactose, the signal transduction factor *GAL3p* in some way relieves the inhibition of the *GAL80p* on *GAL4p* (Apostu & Mackey, 2012; Hawkins & Smolke, 2006; Johnston, 1987). It is not clear how *GAL3p* relieves the inhibitory effect of *GAL80p* on *GAL4p*, but Li et al. (2010) proved that NADP(H) and *Gal3p* can effectively dissociate the *GAL80p-GAL4p* complex. Previous studies have shown that the transcription repressor *Mig1p* can also inhibit the expression of the *GAL* gene in the presence of glucose (Nehlin et al., 1991; Stagoj et al., 2005).

Recently, two articles published by the research group of M. Springer proved that the inhibitory effect of glucose on the *GAL* gene does have two forms (Palme et al., 2021; Ricci-Tam et al., 2021). One is to reduce the number of GAL3p through the competitive binding of glucose and galactose to the transporter (Escalante-Chong et al., 2015), which affects the induction of *GAL* genes. The second is that glucose increases the activity of transcription repressor Mig1p, thereby reducing the expression of the

GAL gene. Using this mechanism, Hayat et al. (2021) introduced auxin-mediated degradation of Mig1p into *S. cerevisiae* to allow inducible expression of pGAL on glucose. In addition, this is a valuable strategy to increase the expression strength of pGAL on glucose. To date, the use of GAL promoter has achieved an efficient synthesis of artemisinic acid (Ro et al., 2006), caffeic acid (P. Zhou et al., 2021), santalols (Zha et al., 2020), and other natural products in *S. cerevisiae*.

pADH2 is derived from *alcohol dehydrogenase II* of *S. cerevisiae*, which is also a widely used inducible promoter. When glucose is present, pADH2 expression is strongly inhibited, and it begins to activate when glucose is depleted. pADH2 has two upstream activation sequences UAS1 and UAS2, among which UAS1 is a 22 bp palindrome sequence that activates pADH2 by binding to TF, *Adr1p*. Studies have shown that changes in the acetylation level of histones with genetic modification are directly involved in changing the chromatin structure of pADH2 and affecting the binding of the promoter to the main transcriptional activator *Adr1p* (Verdone et al., 2002). UAS2 is a GC-rich 20 bp sequence that does not depend on *Adr1p* for glucose regulation of pADH2. The specific regulation of UAS2 is currently unclear, but it is known that those two sequences that work together can activate the expression of pADH2 (de Smidt et al., 2008; Donoviel & Young, 1996). pADH2 was once used to study the efficient synthesis of natural products such as lycopene (Bahieldin et al., 2014) and protopanaxadiol (PPD) (Kim et al., 2018). What is important is that pADH2 does not turn on expression when cells grow and consume glucose. It is only when glucose is exhausted and the cell is in a stable phase with high biomass that pADH2 is expressed efficiently. This mode is ideal for industrial applications (Lee & DaSilva, 2005).

When the environmental copper ion concentration is too high, the *CUP1* gene encodes metallothionein, which tightly chelates with copper ions, thereby maintaining a low level of free copper ions in the cells of *S. cerevisiae* to avoid cell copper poisoning (Fürst et al., 1988). The UAS sequence of pCUP1 contains at least three binding sites for copper ion-dependent TF *Ace1p*. When the concentration of copper ions is high, the N-terminus of *Ace1p* binds to copper ions, and the C-terminus of it binds to a binding site on pCUP1 to activate transcription, resulting in a substantial increase in promoter transcription strength (Laura & Alcide, 2002; Wimalarathna et al., 2012). In recent years, using pCUP1, researchers have conducted studies in *S. cerevisiae* on heterologous substances' biosynthesis, including cyanophycin (Steinle et al., 2008) and isobutanol (Park & Hahn, 2019). In addition, pPHO5 (Wolff et al., 2021) is inhibited in a high-concentration phosphate environment and activated in a low-concentration environment. In reaction to the concentration of ambient oxygen, pDAN1 modifies the strength of its promoter (Cohen et al., 2001). DDI2 (Lin et al., 2018) can efficiently activate expression under the induction of cyanamide, as well as carbon source-dependent promoters such as pSUC2 (Weinhandl et al., 2014). The identification and application of all these inducible promoters have also expanded the team of *S. cerevisiae* promoter elements in metabolic engineering and synthetic biology research.

Studies on the comparison of inducible promoter intensities have also been carried out. For example, Lee & DaSilva (2005) constructed a *LacZ* gene expression vector with three promoters of pADH2, pGAL1, and pCUP1 and introduced them into *S. cerevisiae* to compare the expression strength. As a result, researchers have found that pADH2 under various induction strategies is always better (Lee & DaSilva, 2005). Because gene expression is highly dependent on environmental conditions, it is particularly important to study the intensity changes of different promoters under different conditions, thus, Xiong et al. (2018) discussed differences in the strength of each constitutive or inducible promoter under different stress conditions. Additionally, Peng et al. (2015) used the green fluorescent protein gene as a characterization to compare the intensity differences of different constitutive or inducible promoters under different carbon source conditions.

Heterologous promoters

In addition to endogenous promoters, there are also reports on the use of heterologous natural promoters for expression in *S. cerevisiae*. When a yeast endogenous promoter is used to construct a gene expression cassette, since the promoter sequence is highly consistent, there may be homologous recombination errors, while heterologous promoters will not have such a situation due to their large sequence differences. For example, using pTEF2 and tTEF2 from *Ashbya gossypii* to construct a gene knockout expression cassette can maximize the correct integration rate of homologous recombination (Gueldener et al., 2002). In addition, pCMV from cytomegalovirus has also been shown to be a promoter capable of constitutive expression in *S. cerevisiae* (Becskei et al., 2001; Romero-Santacreu et al., 2010).

Promoter engineering strategies

The applications of natural promoters, whether endogenous or heterologous *S. cerevisiae* promoters have obtained many good results. However, with the rapid development of metabolic engineering and synthetic biology, researchers have become more demanding on promoter elements, hoping to obtain more promoter elements with different regulatory strengths and finer expressions. Thus, a large number of studies have explored modifications and optimizations of promoters. This chapter mainly introduces different promoter engineering strategies studied by researchers in recent years (Fig. 3).

Hybrid

As mentioned earlier, promoters include regulatory components and core components, which determine the transcription intensity and TSS respectively. While natural promoters of *S. cerevisiae* have a limited range of promoter strengths, researchers tried to fuse regulatory components and core components from different promoters (Fig. 3a) and obtained hybrid promoters with a wider range of strengths for finer control of gene expression. There are many options for hybrid promoter elements, and the common selections are endogenous promoter elements of *S. cerevisiae*. Blazeck et al. (2012) selected different core promoter elements to construct hybrid promoter libraries. By fusing the enhancer element which was consisting of tandem repeats or combinations of upstream activation sequences (UAS), a hybrid promoter UAS_{TEF}–UAS_{CIT}–UAS_{CLB}–pGPD was constructed and exhibited a 2.5-fold increase in mRNA levels compared to the endogenous pGPD promoter (Blazeck et al., 2012). Then, the enhanced pGPD promoter maximized the overexpression of the CAD enzyme for itaconic acid production (Blazeck et al., 2014). Similarly, Wang et al. (2018) isolated 10 UAS sequences from strong constitutive promoters and fused them with several core promoters, such as pCYC1, to construct a hybrid promoter library. The activation intensity of $UAS_{ENO2}(3\times)$-pTEF1 in the library was twice as strong as that of pTEF1 which was proven to be a powerful natural promoter of *S. cerevisiae* (Wang et al., 2018). In addition to the UAS sequences of constitutive promoters, Deng et al. (2021) fused the core component of constitutive promoters with the UAS sequence of inducible promoters. By knocking out *GAL1* and *GAL80*, the galactose-induced promoter was transformed into a

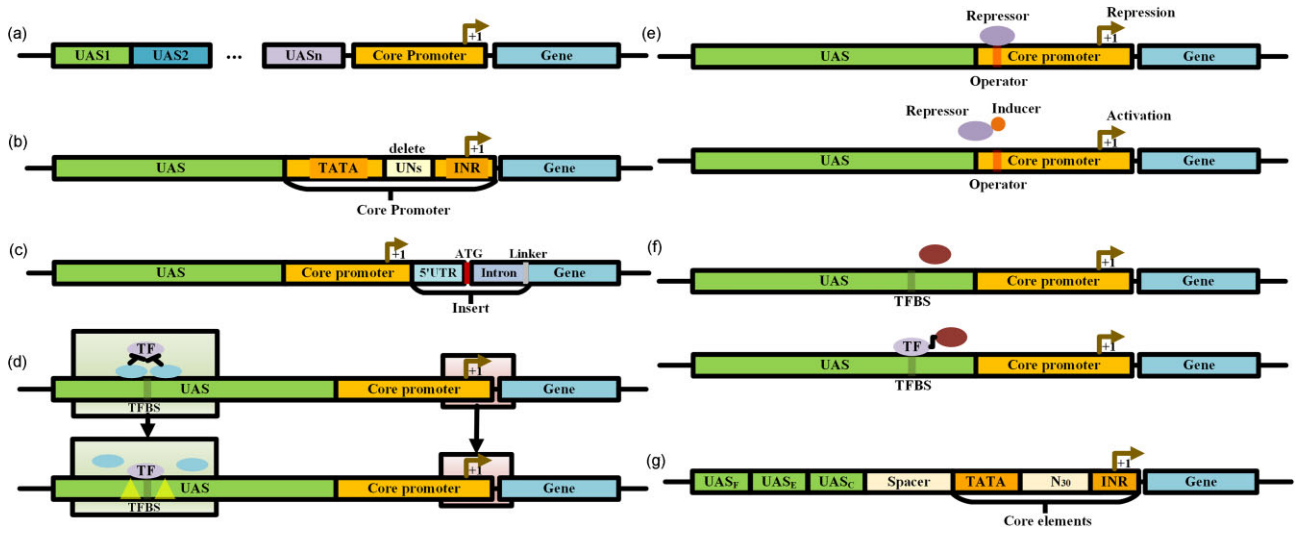

Fig. 3. Promoter modification strategies. (a) Hybrid, which means combinations of UASs from different sources. (b) Truncation, the delete part is shown in bright yellow, and UNs means unnecessary sequences. (c) Intron insertion (Cui et al., 2021), the inserted part includes 5'UTR, ATG, Intron and a necessary linker. (d) Nucleosome removal, blue ovals represent nucleosomes, which hinder the binding of TF to TFBS; yellow triangles represent nucleosome adverse sequences, and the insertion of the sequences around TFBS hinder formation of nucleosomes and promote combination of TF and TFBS. (e) TF-based biosensor. (f) TF modification, the brown ovals represent substances that could affect the activity of promoters; the fusion of TF and the substance can affect the performance of promoters that have the corresponding TFBS. (g) Synthetic (Redden & Alper, 2015). N30 represents 30 random nucleotides.

constitutive promoter that can be expressed efficiently under various carbon sources, and the obtained hybrid promoters UAS_{GAL1}-TDH3/UAS_{GAL1}-TEF1 had a higher expression intensity than the original promoter pGAL1 (Deng et al., 2021).

Other than constructing constitutive hybrid promoters for static regulation, many studies have also used the method to construct inducible hybrid promoters that can dynamically regulate gene expression. The conventional idea is to hybridize the UAS sequence of the endogenous inducible promoter of *S. cerevisiae* with the core sequences of other promoters to construct a new inducible promoter. The UAS sequence of pGAL is commonly used. The UAS_{GAL10}-$Core_{CYC1}$ hybrid promoter was constructed and applied to the study of heterologous protein expression in *S. cerevisiae* (Guarente et al., 1982; Hadiji-Abbes et al., 2009). Bitter & Egan (1988) inserted $UAS_{GAL1–10}$ into the pGPD sequence, constructing a $UAS_{GAL1–10}$-GPD hybrid promoter to guide the expression of cytotoxic human immune interferon-γ (IFN-γ) in *S. cerevisiae*. Besides, Blazeck et al. (2012) hybridized UAS_{GAL1} with different core promoters and constructed a series of hybrid promoters with different inducibility strengths, achieving finer gene expression regulation. Furthermore, promoters that can induce expression in response to tryptophan are also very attractive. Iraqui et al. (1999) hybridized the upstream activation sequence of *aromatic aminotransferase II* gene ARO9 UASaro with pCYC1 core promoter. The study finally got tryptophan-responsive hybrid promoters that help to finely regulate gene expression levels. Apart from the common pGAL and pARO9 promoter elements, Wang et al. (2018) isolated six UAS elements from post-diauxic phase-induced promoters. These elements were hybridized with core promoters of pCYC1, pTEF1, and pGAL1 to construct a hybrid promoter library. Promoters in the library can be automatically induced to a higher expression level after glucose is consumed (Wang et al., 2018).

Apart from the hybridization between endogenous elements of *S. cerevisiae*, hybridizing heterologous elements with endogenous elements has also been proven to be effective. Feng & Marchisio (2021a) connected core promoters from the virus to a short sequence from the endogenous pCYC1 to activate it and placed different TATA boxes in different positions, resulting in 59 hybrid promoters of different intensities, amplifying the promoter library of *S. cerevisiae*. Purvis et al. (1991) hybridized the core element of pPGK1 with the human androgen response sequence to construct a hybrid promoter that can activate expression in response to human androgen. The brief content of this section has been shown in Table 1. These studies demonstrate the great potential of the hybrid strategy. It is simple and effective, and offers more possibilities to construct constitutive promoters or inducible promoters with more diverse strengths.

Random mutations

The hybrid promoter library requires some knowledge of different promoter elements, whereas random mutation can build a larger promoter library with no need to clarify the functions of each promoter element. However, it is necessary to find a fast and efficient screening method.

The first commonly used method of random mutation is error-prone PCR. A mutation library is constructed by randomly introducing mutations into sequences, and then a suitable method is used to screen the mutant promoter to meet a target expression strength. Alper et al. (2005) extended this method to *S. cerevisiae* as early as 2005, based on the successful construction of a bacterial error-prone PCR promoter library. A pTEF1 promoter mutation library was constructed by error-prone PCR, screening 11 mutant promoter sets with a starting strength ranging from 8% to 120% of the original pTEF1 promoter (Nevoigt et al., 2006). On this basis, pGPD1 was replaced with five TEF1 promoter mutants of different strengths, analyzing the effect of glycerol 3-phosphate dehydrogenase activity on glycerol production. Du et al. (2012) used a similar method to construct pPDC1 mutant, pTEF1 mutant, and pENO2 mutant libraries guiding the three-step gene of the xylose utilization pathway and successfully constructed an efficient xylose utilization pathway and a cellobiose utilization pathway. Furthermore, Yuan & Zhao (2013) used the same primers to construct mutation libraries of pENO2 and pPDC1 through error-prone PCR. Combined with directed evolution, they introduced and optimized

Table 1. Summary of Different Promoter Engineering Strategies

Strategy	Theory	Regulation type	Focusing	Result	Reference
Hybrid	Fuse regulatory elements and core components of different promoters	Statically	UAS_{CLB}-UAS_{CIT}-UAS_{TEF}-$Core_{GPD}$	Itaconic acid yield increased 7 times	[Blazeck et al., 2012, Blazeck et al., 2014]
		Dynamically	UAS_{aro}-$Core_{CYC1}$	hybrid promoters induced by tryptophan	[Iraqui et al., 1999]
Random Mutation	Error-prone PCR: Introduce mutations in promoters	Statically	pPDC1/pTEF1/pENO2	more efficient xylose utilization pathway and biobiose utilization pathway	[Du et al., 2012]
		Dynamically	pDAN1	mutants of DAN1 induced by non-strict anaerobic conditions	[Nevoigt et al., 2007]
	Saturated mutation: Preserve conserved region and mutate against spacer region	Statically	pYRP(assembling functional components)	ZWF1 is down-regulated by the engineered promoter	[Jeppsson et al., 2003]
Truncation	Remove unnecessary sequences from natural promoters	Statically	tADH1p	higher production of α-amylase	[Ruohonen et al., 1995]
Intron Inserting	Insert introns in gene expression modules	Statically	pTDH3/TEF1p-RPS25A	Ethanol yield increased by 10%	[Myburgh et al., 2020]
Nucleosome removal	Insert nucleosome adverse sequences in promoters	Statically	pHIS-poly (dA:dT) tracts	70 promoter variants with different intensities	[Raveh-Sadka et al., 2012]
		Dynamically	GAL1-Superbinder	nucleosome removal rate reduced	[Wang et al., 2011]
Chimeric	Insert TFBSs in promoters and introduce site-specific TFs in *S. cerevisiae*	Dynamically	TetR/TetO	A biosensor in response to tetracycline	[Garí et al., 1997]
			FapR/FapO	A biosensor in response to Malonyl-CoA	[Li et al., 2015]
			LacI/LacO	A biosensor in response to lactose or IPTG	[Grilly et al., 2007]
TFs modification	Modify TFs to change the expression of natural promoters	Dynamically	phyA/phyB-GAL4-DNA binding domain; PIF3-GAL4-activation-domain	A biosensor in response to light signal	[Shimizu-Sato et al., 2002]
Synthetic	Design promoters artificially combined with computer technology	Statically	$UAS_{F\text{-}E\text{-}C}$-Core	nine synthetic promoters with similar strength to GPD and shortened length	[Redden & Alper, 2015]
		Dynamically	UAS_{GBS}-Core(GBS = GAL1-derived Gal4p-binding sites)	synthetic promoters induced by galactose	[Redden & Alper, 2015]

heterologous cellobiose utilization pathway, getting significantly higher cellobiose consumption rate (6.41 times) and ethanol production (6.36 times) than those of the parent strain of *S. cerevisiae* (Yuan & Zhao, 2013). Recently, Vaishnav et al. (2022) constructed sequence-to-expression models. Learning a well-predictive deep neural network model from millions of randomly mutated promoters and their measured expression strengths, this model was used to regulate the evolution of mutated sequences. Although the molecular mechanism remains to be elucidated, this study undoubtedly breathes new life into promoter engineering, telling us the possibility of combining different strategies with machine learning (Vaishnav et al., 2022).

In addition to constructing constitutive mutant promoters, some researchers have also used the method to obtain inducible promoters with optimized performance. Nevoigt et al. (2007) isolated two mutants of oxygen-responsive promoter pDAN1

through random mutagenesis, and the mutants could induce expression even under nonstrictly anaerobic conditions. Such mutants are very suitable for gene expression under microaerobic conditions (Nevoigt et al., 2007). Besides, Ingolia & Murray (2007) screened a mutant promoter library by fluorescence-activated cell sorting, and the constructed pheromone inducible promoter pFUS1 mutant induced a decrease in expression level and eliminated the durable response to pheromone.

Apart from error-prone PCR, saturation mutation is also a very effective method that preserves the conserved region and targets the spacer region. Promoter homology through randomizing spacer sequence is lower than that of error-prone PCR, so genetic stability could be higher (Hammer et al., 2006). Because prokaryotic promoters have clear conserved regions, saturation mutations are mainly studied in prokaryotes. *S. cerevisiae* promoters do not have clear spacer sequences, so there are fewer related studies compared with error-prone PCR. Jeppsson et al. (2003) did not conduct saturation mutations on endogenous promoters but designed a new promoter pYRP by assembling the functional elements of *S. cerevisiae* promoters. By changing the separation distance between the assembled elements, they acquired a library containing 37 promoters of different strengths, and the obtained promoters were successfully used to downregulate ZWF1 (Jeppsson et al., 2003). Random mutations can obtain a large number of different promoter sequences in a short period of time, which is a simple method to operate but with a high screening effort. If an efficient screening way can be found, the method would be well worth exploring in depth.

Truncation

Natural promoters of *S. cerevisiae* are usually a few hundred nucleotides in length, and the expression of each gene in *S. cerevisiae* requires the construction of a promoter-gene-terminator expression module. Longer promoters make the construction of biosynthetic pathways in *S. cerevisiae* less efficient. In that case, researchers hope to obtain a promoter that is as short as possible and can guide gene expression normally. It seems feasible to remove unnecessary sequences from natural promoters (Fig. 3b). Some researchers have gained a truncated ADH1 promoter whose activity remains unchanged during the ethanol consumption stage by truncating part of the sequence (Ruohonen et al., 1991, 1995). Improving gene expression efficiency by shortening promoter length requires researchers to have a deeper understanding of the promoter structure, otherwise the desired results may not be obtained. However, promoters of shorter lengths are still worth exploring for long biosynthetic pathways.

Intron insertion

Introns are unique sequences in the genome of eukaryotes. Alternative splicing of corresponding mRNA sequences encoded by introns before translation increases the complexity of gene expression. The correlation between introns and gene expression also attracts researchers to explore ways of using introns to change the strength of the promoter. Yoshimatsu & Nagawa (1994) cloned the intron of *RP51A* and inserted it into different positions of *URA3* and *PGK-lacZ* fusion genes to study the effect of introns on gene expression. Hoshida et al. (2017) proved that introns can promote protein expression. Using pTDH3 and different introns, they construct multiple engineered promoters. Among them, the strongest promoter is approximately 50 times higher than the intensity of pTDH3 (Hoshida et al., 2017). Cui et al. (2021) also constructed a library of engineered promoters with a wider range of intensity by inserting different introns (Fig. 3c). Among them, pGPD + *RPL23A* is the strongest promoter in the library, and its strength is twice that of the natural strong promoter pTPI (Cui et al., 2021). Similar to this method, Myburgh et al. (2020) selected a promoter inserted with *RPS25A* intron and successfully increased ethanol yield. These studies demonstrate the ability of introns to influence promoter expression intensity. Therefore, the combination of a large number of introns with different promoters could provide more possibilities for *S. cerevisiae* promoter libraries.

Nucleosome removal

The nucleosome is a basic repeating unit of chromatin that greatly affects gene expression through differences in number and location (Lam et al., 2008). Studies have shown that GC content can significantly affect the formation of nucleosomes (Schnepf et al., 2020; Trotta, 2022). The density of nucleosomes is negatively correlated with the rate of gene transcription, and the density of nucleosomes bound to promoters in the active regulatory region of the genome is significantly lower than that of other regions (Ercan et al., 2004). So, many researchers studied changing the strength of promoters through manipulation of nucleosomes (Fig. 3d). Xi et al. (2010) proposed a duration hidden Markov model for nucleosome location prediction. Combined with the result of Xi's research, Curran et al. (2014) designed a framework for designing sequences that predict reduced nucleosome affinity and successfully used this method to increase the strength of four natural yeast promoters (pCYC1, pHIS5, pHXT7, and pTEF1). There are poly(dA: dT) tracts in the *S. cerevisiae* genome, which is a sequence that is not conducive to the formation of nucleosomes. Poly(dA: dT) tracts are also significantly enriched in promoters with higher expression (Sharon et al., 2012). So, manipulating the length and composition of poly(dA: dT) tracts can greatly affect the binding of nucleosomes to promoters, changing the activity of promoters. For example, Raveh-Sadka et al. (2012) inserted poly(dA: dT) tracts of different lengths and compositions on both sides of the transcription activator Gcn4p site in pHIS, constructing 70 promoter variants of different strengths. Also, Wang et al. (2011) discussed changes in nucleosome occupancy and mRNA expression under strong or weak induction conditions by inserting a superbinder at GAL1 promoter-1/-2 site, demonstrating that the method can also be applied to inducible promoters. Changing promoter strength by affecting nucleosomes demonstrates that researchers have gained a deeper understanding of promoter structure. This approach may become more mature as nucleosomes are studied more intensively.

Chimeric

Chimeric promoters are mainly constructed through inserting TFBSs into promoters and then introducing TFs that specifically bind to the site into *S. cerevisiae*. TFs and chimeric promoter systems are also known as TF-based biosensors (Mannan et al., 2017) (Fig. 3e). Combination of TFs and specific effectors can regulate the expression intensity of chimeric promoters. By responding to different levels of specific effectors, chimeric promoters can dynamically change the expression intensity to achieve dynamic regulation of metabolic pathways. Many studies have tried to construct chimeric promoters using different elements, and some have incorporated endogenous elements into promoters. For example, Kim et al. (2015) chimerized different numbers of Aro80 TFBSs with pAro9 (Kim et al., 2015), constructing tryptophan-responsive chimeric promoters to fine regulate gene expression levels. Rajkumar et al. (2016) inserted corresponding binding sites of pH-responsive TFs into the upstream activation sequences of pYGP1 and pCCW1 and successfully constructed powerful promoters

induced by a low pH (pH ≤ 3) environment. Applying these chimeric promoters to low pH lactic acid fermentation, the most powerful promoter makes lactic acid production 10 times higher than synthetic production guided by natural strong promoter pTEF1 (Rajkumar et al., 2016). Using the same method, a chimeric promoter induced by glucose starvation and alternate carbon sources was also designed (Rajkumar et al., 2019). However, more research on chimeric promoters has focused on the insertion of heterologous elements. Next, this section will specifically introduce the constructions and applications of several common chimeric promoters.

TetR/TetO

The most widely used heterologous element for constructing chimeric promoters in *S. cerevisiae* is the tetracycline resistance gene regulatory element TetR/TetO which is from bacteria. TetO is the operon of the *Tet* gene, TetR encodes *Tet* repressor, and tetracycline acts as an inducer. When tetracycline is not present, TetR binds to TetO, hindering gene transcription. In the presence of tetracycline, TetR binds to tetracycline, protein conformation changes to break away from the TetO operons on DNA, the hindrance eliminating, and then transcription turns on (Dingermann et al., 1992; Grushka et al., 1992).

In response to tetracycline and tetracycline analogs inhibiting gene expression, the TetO operon sequence was chimeric into endogenous promoters of *S. cerevisiae*, and a chimeric promoter was created by fusing TetR with VP16 from HSV. Different insertion sites and numbers of TetO in promoters will also affect the inhibitory strength of chimeric promoters (Garí et al., 1997). The closer the distance to the TATA box, the better the suppression effect, and the farther the distance, the less obvious the suppression effect (Murphy et al., 2007). Dr. Fanglong Zhao of our research group used *S. cerevisiae* to efficiently synthesize protopanaxadiol. In this study, by inserting TetO into the promoter of lanosterol synthase *ERG7* and using the ACS1p-TetR-ADH2t module to express TetR, the constructed chimeric promoters successfully inhibited the synthesis of byproduct lanosterol, increasing the PPD output from 432.6 mg/L to 512.3 mg/L (Zhao et al., 2018). In addition to using original components, researchers also modified components to achieve different regulatory effects. Cuperus et al. (2015) identified variants of TetO and used these variants to replace the original one, generating more than 100-fold gene expression. *S. cerevisiae* strains with high lycopene production were selected by combining these variants to guide the expression of CrtE, CrtB, and CrtI (Cuperus et al., 2015). Peng et al. (2022) designed a tetracycline-mediated circuit to minimize strain metabolic burden by combining it with a 37°C-sensing circuit to relieve glucose repression of pGAL during biological processes. The constructed gene circuit finally increased the production of terpenoid nerolidol by 44%, reaching 2.54 g/L in flask cultivation. This study provides a favorable reference for more precise dynamic regulation, and also allows us to see broad prospects for the combination of different gene circuits. (Peng et al., 2022).

FapR/FapO

The FapR/FapO system comes from *Bacillus subtilis*, and FapO is the binding site of the FapR protein located on the fatty acid synthesis promoter. The N-terminal domain of FapR protein specifically binds to FapO and the C-terminus particularly binds to malonyl-CoA. When the concentration of malonyl-CoA is low, FapR binds to FapO, and steric hindrance inhibits the promoter from opening expression. As the concentration gradually increases, FapR binds to malonyl-CoA, and the N-terminal conformation changes to break away from the FapO binding site, making promoter inhibition disappear; hence, downstream genes start transcription (Schujman et al., 2003, 2006).

The FapR/FapO system was first applied to *Escherichia coli* (Xu et al., 2014), and other prokaryotic cells, constructing engineered bacteria with high production of malonyl-CoA derivatives such as fatty acids (Xu et al., 2014). In recent years, the system has gradually been applied to eukaryotic cells, including *Pichia pastoris* (Wen, Tian, Liu, et al., 2020; Wen, Tian, Xu, et al., 2020) and *S. cerevisiae*. Li et al. (2015) chose the strong promoter pTEF1 to direct the expression of FapR, and used pGPM1 to control the expression of fluorescent protein tdTomato, then inserted FapO into the upstream position of the pGPM1 TATA box, for the first time by applying the FapR/FapO system into *S. cerevisiae* cells. By combining the sensor with the whole genome cDNA overexpression library, they screened yeast strains with high production of malonyl-CoA. Furthermore, two favorable target genes, *PMP1* and *TPI1*, were found, which ultimately increased the production of 3-HP, a derivative product of malonyl-CoA (Li et al., 2015). Similarly, using the FapR/FapO system, David et al. (2016) increased the yield of 3-HP to 1 g/L by dynamically regulating the synthesis pathway of 3-HP and the competitive pathway *FAS1*.

LacI/LacO

The LacI/LacO system comes from bacterial genes required for lactose utilization. When the repressor LacI binds to the operon LacO on promoters, the transcription of the lactose utilization gene is inhibited. When lactose or its replacement inducer isopropyl-beta-D-thiogalactopyranoside (IPTG) is present, the repressor LacI is allosteric and detaches from the LacO operon site, canceling gene transcription repression (Lewis, 2005). Grilly et al. (2007) integrated mammalian-enhanced LacI into *S. cerevisiae* pADH1, constructing an ADH1i promoter that was inhibited in the absence of IPTG and could drive gene expression after IPTG was added.

In addition to designing a pure LacO chimeric promoter, more studies have combined the LacI/LacO system with other regulatory systems to construct a multiple-response system. For example, Ellis et al. (2009) chose tetO chimeric promoters constructed by themselves to drive the expression of LacI. Based on pGAL1, a promoter $P_{OR\text{-}LT}$ containing LacO and tetO operons was designed to drive the expression of yEGFP. The final constructed synthetic gene network can adjust the expression intensity of yEGFP by changing the concentration of tetracycline and IPTG (Ellis et al., 2009). Mazumder & McMillen (2014) constructed a chimeric promoter that can be activated by testosterone-responsive androgen receptors and inhibited by LacI by adding five steroid hormone-responsive elements and one LacO operon upstream or downstream of the pCYC1's TATA box, respectively. Through the joint regulation of testosterone and IPTG, the output signal curve of the promoter can be adjusted over a wide range (Mazumder & McMillen, 2014). Similarly, Gnugge et al. (2016) also constructed a dual-mode promoter by inserting (tetO)2 and (LacO)2 upstream or downstream of the pCYC1's TATA box.

Other chimeric promoters

In addition to the three types of regulatory elements above, there are also other regulatory elements used. For example, xylose-responsive TFs (XylR) were introduced into *S. cerevisiae*, and the XylO operon was chimerized to bind to XylR in natural promoters, constructing a biosensor that responds to the concentration of xylose (Teo & Chang, 2015; Wei et al., 2020). By introducing fatty acid-responsive repressor protein (FadR) from *E. coli* or *Vibrio cholera*,

and chimerizing the FadO operon that can bind to FadR in natural promoters, a biosensor that responds to fatty acid concentration can be constructed (Teo & Chang, 2014; Teo et al., 2013). The introduction of SAM-responsive repressor protein (MetJ) from *E. coli* and the MetO operon capable of binding to MetJ in promoters can construct a biosensor that responds to the concentration of SAM (Umeyama et al., 2013). Zhou et al. (2021) successfully downregulated gene expression by inserting *E. coli* cis-element marO into native promoters of *S. cerevisiae ERG1* and *ERG11* genes, resulting in a 4.9-fold increase in squalene or a 4.8-fold increase in lanosterol. Mclsaac et al. (2014) inserted 6 Zif268 binding sequences into pGAL1 and constructed an artificial TF, Z3EV that can bind to Zif268 so the system can adjust the expression intensity in response to the level of β-estradiol. Similarly, there was a study on inserting synthetic SKN7 response element SSRE into pMEL1 of *S. cerevisiae* to regulate expression, and successfully constructed an *Arabidopsis thaliana* signaling system (Chen & Weiss, 2005). As more elements are discovered, chimeric promoters that respond to specific effectors have a wider application, gradually realizing the need of researchers for precise regulation. It can be said that this strategy has a very promising future.

TFs modification

Some studies have affected the expression of natural promoters through the design and modification of TFs (Fig. 3f). This strategy does not directly modify a promoter itself, but changes the expression strength of the promoter and the response to specific signals by modifying substances that can affect the activity of the promoter. Shimizu-Sato et al. (2002) fused PIF3 protein which interacts with phytochrome under specific conditions with the GAL4-activation domain (PIF3-GAD), and this fusion protein enabled promoters that had GAL4-DNA binding sites to switch and regulate expression in response to light signals (Shimizu-Sato et al., 2002). Similarly, by fusing the DNA binding domain of GAL4, the hormone-binding domain of human estrogen receptor, and the activation domain of viral protein 16 (VP16), the obtained TF was able to induce the expression of a promoter that contains the GAL4-DNA binding domain in the presence of β-estradiol (Louvion et al., 1993; McIsaac et al., 2011). This strategy achieves targeted modification of promoter expression in an indirect way. Combined with the hot research on chimeric promoters, as an important part of chimeric elements, it is believed that modified TFs can play a greater role in the near future.

Synthetic

Research on natural promoters of *S. cerevisiae* has greatly promoted the development of metabolic engineering and synthetic biology. However, the widespread use of natural promoters may lead to homologous recombination. Moreover, modified promoters still depend on endogenous structures of *S. cerevisiae*, and few heterologous promoters play a role in *S. cerevisiae*. So, in recent years, researchers began to think about expanding the *S. cerevisiae* promoter library by artificially designing promoters. Curran et al. (2014) used a model-guided approach designed to predict nucleosome affinity reduction sequences by inserting random spacer sequences between the common glycolytic TFBSs, acquiring six pure synthetic promoters. Redden & Alper et al. (2015) used oligonucleotides to randomly synthesize core components of promoters and hybridized them with 10 bp UAS sequences. Through a series of screenings, nine promoters with constitutive strength comparable to pGPD and shortened length were identified from a pool of 15 million candidates, which minimized the burden of DNA (Fig. 3g) (Redden & Alper, 2015). Since the assembly of random sequences requires extensive screening of synthetic promoters, Kotopka & Smolke (2020) proposed a model-based sequence design method. Fluorescence-activated cell sorting-SEQ (a massively parallel reporter analysis, MPRAs) was performed on two libraries containing 675 000 constitutive promoters and 327 000 inducible promoters. Using these data sets, a convolutional neural network model was trained to predict promoter activity with high accuracy. As a result, researchers successfully constructed large, sequence-diversified promoter sets containing constitutive promoters and inducible promoters respectively (Kotopka & Smolke, 2020). This research allows the world to see that a combination of machine learning and biotechnology can greatly expand the possibilities of biological research. Besides, *E. coli* (Jin et al., 2019) and *Yarrowia lipolytica* (Liu et al., 2020) have also studied artificial synthetic promoters. With the joint development of computational science and synthetic biology, the prospect of artificially designing promoter elements will be very bright. All the promoter engineering strategies above are organized in Table 1 for comparison and reference.

Combination of Different Strategies

To build functionally optimized promoters, various promoter engineering strategies are used. Most researchers limit their studies to only one promoter engineering strategy, so comprehensive applications of different strategies do not currently receive much attention. However, different strategies have different research focuses. If they are applied comprehensively, these may inject more vitalities into the promoter family and bring more possibilities.

In recent years, there have been some studies trying to combine different strategies. To further verify the versatility of artificially synthesized core promoter elements, Redden & Alper et al. (2015) hybridized it with the smallest galactose-induced UAS element and successfully constructed galactose-induced synthetic promoters. Zhang et al. (2016) modified TRX2, the target promoter of Yap1's natural regulatory pathway, chimerizing multiple Yap1p binding sites and hybridizing the UAS sequence of TRX2 and constructed a series of promoters with different strengths that are induced by diamide. Their activation strengths expanded with the increased numbers of UAS (Zhang et al., 2016). Leavitt et al. (2016) hybridized UASaro with Leu_{min} core promoter and expressed a mutant Aro80 TF at the same time, constructing tryptophan-responsive promoters that were more conducive to fine regulate gene expression levels. Decoene et al. (2019) truncated the 176 bp core promoter of pTEF1 as a starting point based on previous research on pTEF1 (Blazeck et al., 2012) and screened out the smallest core sequence capable of expressing the protein. Then, they combined different methods such as mutagenesis of core sequence and hybridization of upstream activation elements, obtaining a series of short promoters with different initiation strengths (Decoene et al., 2019).

Relatively more studies combined chimeric promoters with other promoter engineering strategies, which is also a direction to tap the greater potential of engineering promoters. For TetR/TetO system, Gossen et al. (1995) got a reverse TetR (rTetR) through mutation. When doxycycline (a tetracycline analog) was present, the inhibitory effect of TetO-controlled gene expression increased, which was opposite to the effect of wild-type repressor. Using TetR and rTetR, Gemma et al. (1998) constructed a dual activator/repressor system regulated by tetracycline, allowing strict expression control. Ellis et al. (2009) retained the fixed motif of pGAL, randomly mutated nucleotides around TetO operon in promoters, and finally obtained a library of 20 regulatory promoters with

different expression levels. Recently, more precise expression regulation has gradually become a reality. A study constructed a powerful promoter that was repressed by TetR and induced by tetracycline and its analog anhydrotetracycline. Then, by creating an autorepression loop, designing a chimera of TetR and an active yeast repressor Tup1, they constructed controllers that precisely set expression levels under different circumstances, at different times, and different cell cycles (Azizoğlu et al., 2021). Chen et al. (2018) inserted FapO into the UAS_{TEF1}-$CORE_{GAL1}$ hybrid promoter for the fapR/fapO system, constructing a sensor with higher sensitivity to malonyl-CoA, then used the sensor to screen the phosphorylation site of acetyl-CoA carboxylase ACC1. This proves the effectiveness of the sensor's high-throughput screening function. An important factor in evaluating sensor performance is the dynamic range of sensor response. So, Dabirian et al. (2019) selected several different promoters and constructed a sensor promoter library with different response ranges by changing FapO insertion sites and numbers and fusing different transcription inhibitors. Besides these studies, Qiu et al. (2020) successfully constructed biosensors for repressive regulation by fusing FapR with the GAL4 activation domain. Thus, as the concentration of malonyl-CoA increased, the expression intensity of the chimeric promoter gradually decreased (Qiu et al., 2020). This study makes the FapR/FapO system have more possible application directions.

It can be seen from the above research that the combinations of different promoter engineering strategies make up for the limitations of a single strategy and expands the strength range and transcriptional regulation precision of engineered promoters, which brings bright prospects for further optimization of promoter elements. Until now, there are not many studies on the combination of different promoter engineering strategies. This review aims to increase the attention of researchers combining different strategies, by summarizing different promoter engineering strategies. For example, chimeric promoters are modifications based on *S. cerevisiae* native promoters. If we can use artificially designed and synthesized strong promoters to chimerize different TFBSs, and combine machine learning to optimize engineered promoters, we may be able to acquire powerful artificial chimeric promoters for metabolic engineering. Alternatively, by fusing two TFs with different responders, and chimerizing their corresponding binding sites in promoters, it may be possible to construct a dual-responsive promoter that changes expression according to changes in different signals, and this may be able to further improve the regulatable degree and regulation precision of promoters in metabolic engineering. In conclusion, the combined use of different promoter engineering strategies has great potential for development, and if more researches work, it will surely further promote the development of metabolic engineering and synthetic biology.

Conclusion

Due to the importance of promoters in gene transcription, many identifications and analyses of natural promoters and modifications of them have been carried out for decades. The application of well-functioning promoters to metabolic engineering or synthetic biology can greatly promote research in the field. On account of the limitations of natural promoters, promoter engineering has developed rapidly. The primary goal of modifying promoters used for static regulation is to obtain constitutive promoters of varying strengths. The rational use of these promoters in different metabolic pathways is conducive to the fine regulation of metabolic flux. The engineered promoters used for dynamic regulation make the metabolic process more controllable. The rational addition of inducers results in an inducible promoter with high response sensitivity and a wide response range, allowing metabolism to flow dynamically to target products. Apart from the single use of strategies shown above, combining different strategies gives promoter engineering more possibilities. Combining synthetic promoters to construct TF-based biosensors, using nucleosome removal, intron insertion, and other means to improve the transcriptional strength of chimeric promoters, or fusing different TFs to construct dual-responsive chimeric promoters, the combinations of different strategies have so many possibilities, waiting for researchers to explore. The comprehensive application of different strategies will provide promising prospects for further optimizing promoter elements. In the era of artificial intelligence and big data, combining more computer technology may be able to create surprising results.

Funding

This work was supported by the Key-Area Research and Development Program of Guangdong Province (2020B0303070002) and the National Key Research and Development Program of China (No. 2019YFA0905100).

Author Contributions

S.H. retrieved all the data and wrote the first draft of the manuscript. Z.Z. edited and improved the quality of review article. W.L. performed revision, reading, and approval of the final submitted manuscript. All authors listed have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals

Not applicable.

Informed consent

Not applicable.

Additional information

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