Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-019-10172-y

MINI-REVIEW

Promoter engineering strategies for the overproduction of valuable metabolites in microbes

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Received: 16 July 2019 / Revised: 4 October 2019 / Accepted: 8 October 2019

Abstract

Promoter engineering is an enabling technology in metabolic engineering and synthetic biology. As an indispensable part of synthetic biology, the promoter is a key factor in regulating genetic circuits and in coordinating multi-gene biosynthetic pathways. In this review, we summarized the recent progresses in promoter engineering in microbes. Specifically, the endogenous promoters are firstly discussed, followed by the statement of the influence of nucleotides exchange on the strength of promoters explored by site-selective mutagenesis. We then introduced the promoter libraries with a wide range of strength, which are constructed focusing on core promoter regions and upstream activating sequences by rational designs. Finally, the application of promoter libraries in the optimization of multi-gene metabolic pathways for high-yield production of metabolites was illustrated with a couple of recent examples.

Keywords Promoter library · Transcription element · Metabolic engineering · Biosensor · Mutagenesis

Introduction

The balance of cellular metabolic flux relies on the coordination of interwoven metabolites, enzymes, and regulatory factors. Although a set of approaches have been adopted to enhance the productivity of industrial microorganisms such as mutagenesis (Kodym and Afza 2003; Zhang et al. 2014) and evolution technology, the boosted degree of products with interests cannot always meet the demands. Given the fact that the target of product is always catalyzed by multiple processes with the function of different responsible enzymes, further improvement of the favored product formation requires rational design of the metabolic pathway via metabolic engineering (Clomburg and Gonzalez 2010; Huang et al. 2016, 2018; Redden et al. 2014; Zhou et al. 2017b). One major strategy to investigate the full combinatorial libraries of each gene in the metabolic pathways is the use of the regulatory element of promoter. Promoter engineering provides as an efficient strategy to regulate the promoters with diverse strengths and functions, and has been recognized as a useful tool to precisely control and regulate the gene expression. However, the simple construction of promoter libraries, which is the classic method for promoter engineering, is hard to satisfy the efficient and precise co-expression of multiple genes in metabolic networks. Hence, more and more dynamic transcription regulations (Mannan et al. 2017; Moser et al. 2018) were exploited by the promoter engineering combined with introducing the metabolic flux analysis, mathematic model simulation, or intermediate-biosensor (Oyarzún and Stan 2013). Especially, the biosensor is high potential to auto-regulate the metabolic network for the feature of the indictor detective, simple equipment, and fast reflection.

Transcription control of specific genes is a direct and effective method to regulate metabolic flux (Blazeck and Alper 2013). Promoters design with various strengths could realize the fine-tune of gene expression. Therefore, a series of researches focus on construction of promoters and their modification by sequence randomization, promoter hybridization, and mathematic prediction. Besides transcription, metabolic flux could also be systematically optimized to enhance the production of target products by regulation of the level of translation, mainly focusing on the modification of ribosome

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Published online: 19 October 2019

binding site (RBS), 5′-untranslated region (5′ UTR), and RNase III cleavage of mRNA transcripts (Babiskin and Smolke 2011). It is notable that none of RBS, 5′ UTR, and RNase III cleavage of mRNA transcripts is a promoter component, although they are engineered simultaneously when engineering promoters in many cases (Babiskin and Smolke 2011; Kosuri et al. 2013).

The purpose of this review is to provide strategies of promoter engineering in metabolic engineering, especially in bacteria and yeasts. First, the application of endogenous promoters and the necessity of constructing promoter libraries were illustrated. After that, methods for promoter library construction by site-selective mutagenesis were introduced. Finally, a few applications of promoter engineering combined with the metabolic balance strategy for valuable metabolites production were outlined.

Endogenous promoters of microbials

Microbial genomes, which contain a variety of endogenous promoters, are regarded as huge reservoirs for promoter engineering and metabolic pathway engineering. So far, a number of endogenous promoters have been applied in hosts for overexpression of various enzymes, which are involved in the metabolic pathways of valuable metabolites production. For example, six endogenous promoters (P_{groE}, P_{cdd}, P_{rplK}, P_{sspE}, P_{sacA}, and P_{sacB}) from the genome of *B. subtilis* THY-7 have been constructed distinctively and used for the expression of surfactin synthase, among which the highest yield of 1.09 g/L surfaction was achieved by using the P_{sacB} promoter. Moreover, endogenous promoters with special characteristics can be applied in a conditional medium for high-level production. Lara et al. (2017) found that a *Vitreoscilla* hemoglobin promoter (P_{vgb}) and a set of microaerobic endogenous promoters (P_{adhE}, $P_{adhEred}$, P_{nark}, $P_{narkred}$, P_{kat}, and P_{vgb}) were sensitive to the oxygen concentration and therefore affected the expression of FMN-binding fluorescent protein at oxygen-limited cultures. The application of promoters P_{adhE}, P_{pfl}, and P_{vgb} in *E. coli* under oxygen-limited conditions resulted in three highest transcription levels of the enzymes of interest.

Endogenous promoters can also be screened by quantification of relevant metabolites or certain indicators that are able to monitor the metabolic flux. In the case of regulation of the *N*-acetylglucosamine (GlcNAc) metabolic flux, three endogenous promoters with different strengths, including a high strength constitutive promoter P_{43}, a medium strength xylose inducible promoter P_{xylA}, and a low strength IPTG inducible promoter P_{grac}, were used for the transcription level of genes *glcK* and *pgi*, which are involved in production of GlcNAc. The highest GlcNAc titer reached 35.12 g/L in a 3-L fed-batch bioreactor when the medium strength promoter P_{xylA} was chosen to drive both *glcK* and *pgi* genes (Ling et al. 2017).

Endogenous yeast promoters, with the assistance of transcription elements, showed satisfying strengths and practicalities in promoter engineering. The commonly used endogenous promoters of *S. cerevisiae* include constitutive promoters P_{TEF1} (Gatignol et al. 1990), P_{ADH1} (Denis et al. 1983), P_{TPI1}, P_{HXT7} (Diderich et al. 1999; Reifenberger et al. 2010), P_{TDH3} (P_{GPD}) (Bitter and Egan 1984), P_{PGK1} (Ogden et al. 1986), P_{PYK1} (Nishizawa et al. 1989), and inducible promoters P_{GAL1} and P_{GAL10} (Adams 1972; John and Davis 1981; Laughon and Gesteland 1982). As an application of endogenous promoters in metabolic pathways, fourteen endogenous promoters were rapidly assembled to replace the original promoters of five key genes (*CrtE*, *CrtB*, *CrtZ*, *CrtI*, and *CrtY*) that are in charge of the zeaxanthin biosynthesis (Sun et al. 2012). These fourteen promoters were classified into three groups: high strength group (P_{TEF1} and P_{TPI1}), medium strength group (P_{GPM1}, P_{TDH3}, P_{FBA1}, P_{PDC1}, P_{ENO2}, P_{PYK1}, and P_{TEF2}), and low strength group (P_{PGK1}, P_{HXT7}, P_{TDH2}, P_{ADH1}, and P_{PGI1}) (Shao et al. 2009). Different combinations of these promoters for driving the transcription of *CrtE*, *CrtB*, *CrtZ*, *CrtI*, and *CrtY* resulted in significant differences of zeaxanthin yield, and high yields were achieved when *CrtE*, *CrtB*, *CrtZ*, *CrtI*, and *CrtY* were driven under the promoters of P_{PDC1}, P_{TPI1}, P_{FBA1}, P_{GPM1}, and P_{GPD}, respectively.

Compared with prokaryotic promoters, endogenous yeast promoters show a higher strength, which facilitate their applications in current metabolic engineering. Since the balance in metabolic flux is of great significant for the improved production of interested products, combination of endogenous promoters with different strengths was always applied (Latimer et al. 2014).

Promoter strategy of site-selective mutagenesis

Although endogenous promoters exhibit appropriate strengths, it is still necessary to obtain synthetic promoters to further fine-tuned gene transcription in metabolic networks (Liu et al. 2015; König et al. 2019). During the production of chemicals by microbial factories, generating a dynamic range of transcriptional levels is necessary to achieve diverse metabolic engineering goals. Therefore, promoter libraries with fine-tuned strengths were required to express target genes in a controlled fashion to optimize metabolic network (Zhang et al. 2018b).

Site-selective mutagenesis by error-prone PCR

Site-selective mutagenesis of promoters has been applied for the construction of many promoter libraries and been contributed to the identification of critical regions in promoters by random mutation. Error-prone PCR (ep-PCR) is a classic

method for site-selective mutagenesis, which introduces mutations into DNA fragment at a high concentration of Mn^{2+} in the PCR reaction mixture (Kagiya et al. 2005). Because a large number of variants are generated by ep-PCR, a high-throughput screening method based on a reporter-like green fluorescent protein (GFP) is always employed to characterize the strengths of mutant promoters. Qin et al. (2011) characterized approximately 30,000 P_{GAP} variants through ep-PCR by the reporter yEGFP, and 33 promoter mutants were selected to constitute a promoter library, the activity range of which were between 0.6- and 19.6-fold to the wild-type promoter (Qin et al. 2011). ep-PCR can be applied to exploit the effect of base sites on promoter strength. For instance, a *Saccharomyces cerevisiae* TEF promoter library was constructed by ep-PCR with an activity range between 8 and 120% of the wild-type promoter, and the sequences of mutated promoters showed a continuous string of 10 adenines generated by deleting the single guanine at −251 position of P_{TEF1} (Nevoigt et al. 2006).

Site-selective mutagenesis by saturation mutagenesis

Except for ep-PCR, saturation mutagenesis is always applied for promoter engineering with all possible nucleotides substitution at the specific region (Rui et al. 2018; Marjan et al. 2007; Patwardhan et al. 2009). Rud et al. (2006) developed a synthetic core promoter library by saturation mutagenesis at the non-conserved sequences of 16S rRNA promoters from the *Lactobacillus plantarum* WCFS1 genome, and the relationship between the mutations of the target sequences and the promoter strengths was revealed.

The obtained promoter library enabled fine-tuning of stable gene expression in *L. plantarum* for various applications. These methods introduced various activities of the promoters, which provide huge numbers of mutations to study the structures of promoters. It was reported that a promoter is composed of a core region and a *cis*-regulatory module (CRM). The core region, where RNA polymerase is recruited to initiate transcription, is recognized as the most important component of a promoter, while CRM alone is non-functional for transcription initiation (Blazeck and Alper 2013; Juven and Kadonaga 2010). And it is for boarder application to introduce the engineered promoter modification into the microbe genome by Crispr-cas9, a new developed toolbox (Blazeck and Alper 2013; Donohoue et al. 2018), while modified in the plasmid by random mutation for screening more promoter.

The structure of the prokaryotic core promoter (Fig. 1a) is relatively uncomplicated and definite. With an example of promoter synthesis (Marjan et al. 2007), the transcription start site (TSS), where the first transcribed base in transcription process, is defined as +1 site of a promoter. A few researches focused on investigating the effect of the end's length of TSSs on multi-gene assembly by analyzing the influences of truncated, replaced, or elongated TSSs on promoters (Liu et al. 2016). Furthermore, as conserved sequences in the core region, the nucleotides in −35 box and −10 box show greater influences on the promoter strength compared with the non-conserved regions. The −35 box shares high identity with the TTGACA sequence, while the −10 box is conserved with the canonical TATAAT sequence. A large number of synthetic promoter libraries have been constructed by modifying the region at −35 box to −10 box. Marjan et al. (2007) constructed a promoter library with sequences around −35 box and −10 box modified by saturation mutagenesis and the result showed that the promoters exhibited more than 300-fold differences in transcription abilities. In another example, the influence of the sequences between the −35 and −10 box was studied by saturation mutagenesis of $P_{porin,}$ and a constitutive promoter library was obtained with a 310-fold variation in transcriptional activity in *Halomonas* TD01 strain (Li et al. 2016). CRMs in prokaryotic promoters can be classified as Upstream (UP) elements and operons (only in inducible promoters). UP element is a DNA sequence located upstream of a promoter that interacts with the α-subunit of RNA polymerase (RNAP). UP element can affect transcription by altering the binding of RNA polymerase to DNA (Yan and Fong 2017).

In contrast to the definite characterization of promoters in prokaryote, there is still a lack of systematic clarification in yeast promoter (Fig. 1b). For approximately 19% of yeast promoters (Basehoar et al. 2004), TATA box is the major determinant of promoter activity (Lubliner et al. 2013). The location, orientation, and flanking bases of TATA box critically affect the function of promoters (Lubliner et al. 2015).

Upstream CRM in yeast contains transcription factor binding site (TFBS). CRMs, classified as upstream activating sequences (UAS) and upstream repressible sequences (URS), localized the transcription factors (TFs) covering upstream of the core element in the eukaryon (Portela et al. 2017). Deletion or insertion of CRMs (more specifically in predicted TFBSs) resulted in promoter activity variations (Hartner et al. 2008; Ruth et al. 2010; Vogl et al. 2014). The changes of location, number, orientation, affinity, and organization of TFBSs have been demonstrated to exhibit influence on gene expression level. In addition, sequences that surround CRMs also exert significant effects on gene expression. The overall effects of TFBSs are weakening as their distance from the gene start increases (Sharon and Al 2012). Diversification to TFBSs embellishes the effect of TFs intuitively. For instance, through the analysis of Aro80 binding site ($CCGN_7CCG$), repeats of Aro80 binding half site (CCG) provided a better affinity to the promoter. And a synthetic U_4C_{ARO9} promoter, composed of four repeats of Aro80 binding half site and Aro9 core promoter element, was applied to express *alsS* and *alsD* genes for acetoin production, resulting in an increase in acetoin titers in *S. cerevisiae* (Kim et al. 2015).

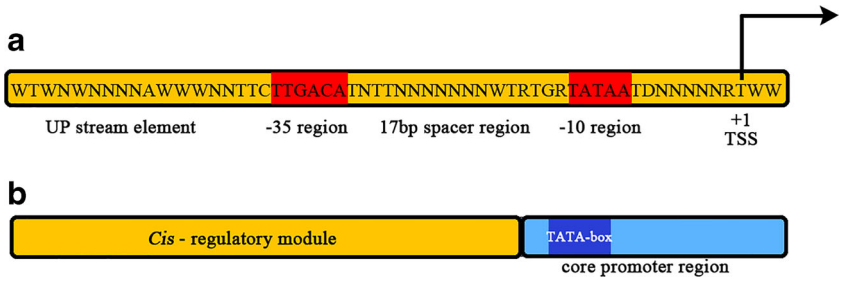

Fig. 1 Structure of *E. coli* promoter (**a**) and yeast promoter (**b**)

During ep-PCR, mutations are randomly taking place and only the mutation ratio of the PCR region is known, whereas saturation mutagenesis characterizes specified sites in the promoter structure. However, as the library is enormous, a high-throughput screening system, as fluorescence-activated cell sorting technology, has to be set up for the mutagenesis is lack of efficiency and accuracy (Jha et al. 2014).

Site-selective mutagenesis based on directional design

With the aids of analysis of a large number of promoter samples (Blount et al. 2012; Brewster et al. 2012; Yu et al. 2016) and the specific site mutations, the features of promoter sequences would be summarized and the effects of single nucleotides in promoters on transcription are revealed, facilitating further study for design of the promoters. Reassembling known functional regions to create new hybrid promoters shall be another effective strategy for promoter library construction (Blazeck and Alper 2013; Blazeck et al. 2011, 2012, 2013, 2014). Due to the limited length of a promoter sequence, it is feasible to enumerate the mutation to every nucleotide position upregulating or downregulating the promoter strength. After the clarity of the critical promoter site impact, a basis has been provided for a directional regulation or further data model construction. We definite the direct modification based on single base function as directional design. The effects of all possible single-nucleotide mutations for −45 to +25 region of the three bacteriophage promoters (P_{T7}, P_{T3}, or P_{SP6}) were assayed quantitatively via nucleotide synthesis on programmable microarray (Patwardhan et al. 2009). Without a clear understanding of the features of elements that constitute promoters in this strain, such as −10 and −35 elements (Österberg et al. 2011), it is impossible to develop useful promoter library in a rational approach (Alper et al. 2005; Rud et al. 2006; Siegl et al. 2013). Although modifications of the core promoter sequence determined the basic strength of the promoters, but the induction or repression profiles was unchanged (Vogl et al. 2014). Since it is already known that A/T rich is conducive to the promoter strength, A/T content is designed to be increased via purposeful point mutations (Lubliner et al. 2013). However, in some cases, replacement of adenine triplets with cytosines may disturb such A/T rich sequences; it is therefore more efficient to characterize the long prokaryote promoters instead of nucleotide enumeration. Starting with the AOX1 wild-type sequence consisting of *cis*-regulatory module (CRM), core promoter, and 5′ UTR, three adjacent wild-type nucleotides were replaced by cytosines (CCC) or adenines (AAA), at one time for each construction. A total of 130 different constructs were created to cover the whole core promoter region (Rui et al. 2018). Modification and cooperation of the elements that combine a whole promoter can be carried out to obtain promoters with precise function and strength.

Mathematical calculation and in silico methods are always applied during promoter engineering, to predict the effects of various promoters on expression levels when the structure and effect of the promoter are clearly correlated by site-selective mutagenesis. Based on energy matrix (Kinney et al. 2010), a synthetic promoter library was generated in *E. coli*, which contains 20 rationally designed promoters with strengths ranging from 0.8 to 100% compared with the original *trc* promoter. The above promoter library was used for combinatorial expression of single CO_2 transport gene (*sbtA* or *bicA*) and single CO_2 fixation gene (*ppc* or *pck*). As a result, the succinate production was increased by 37.5% with a yield of 89.4 g/L (Yu et al. 2016). Another example referred to a set of 18 unique TFBSs was designed for *E. coli* RNA polymerase σ^{70} holoenzyme by using a model of sequence-dependent binding energy calculation combined with a thermodynamic model of transcription to produce a targeted level of gene expression (Brewster et al. 2012). With the analysis of promoter sequence, rational design of the target promoters is available to build the promoter library. The combinations of CRM are interpreted primarily through simple protein-DNA and protein-protein interactions with complicated biochemical reactions (Gertz et al. 2009). After performing an in silico analysis of P_{AOX1} and constructing a deletion library to identify the putative TFBSs, Hartner et al. (2008) doubled the putative Mat1-Mc-binding site which resulted in a 3-fold improvement of promoter activity compared with the wild-type in presence of methanol. This strategy aims to predict the activity of novel combinations of *cis*-regulatory site, and to gain insight into the mechanisms that generate diverse expression levels from different arrangements of the same *cis*-regulatory sites.

Promoter strategy of hybrid promoter

The hybrid promoter strategy, which is primarily based on the characterization of the element function, is a common strategy for rational design. Various structural parts of the desired function are linked together to endow multi-functions on new hybrid promoters. This strategy has been widely applied to insert the inducibility into promoters, such as *trc* (Jung et al. 2010), *tac* (Boer et al. 1983), *mac* (Vidal-Ingigliardi and Raibaud 1985), and *pac* (Shibui et al. 1988) promoter. Appending an operon on a promoter is another strategy of hybrid modification, which regulates statically the transcription level by the addition of exogenous inducer (Zhou et al. 2017b). Inducible promoters reduce the toxicity and burden of metabolite to cells prominently, while the dose-dependent regulation works indispensably to optimize the metabolic network. An artificial IPTG-inducible promoter P_{Pg2}, which was constructed from the fusion of a native promoter P_{groE} and operon *lacO*, replaced the native promoter P_{srfA}, to achieve the engineered strain, resulting in an increased surfactin titer at 5.98 g/L (Jiao et al. 2017). In addition to the commonly used IPTG-inducible factors, new inducible factors can be obtained through hybrid strategies. Horbal et al. (2014) fused the *Pseudomonas putida* cumate degradation operon and synthetic promoter P_{P21} to develop a new cumate (*p*-isopropylbenzoic acid)-inducible gene switch based on the CymR regulator in actinobacteria. As the extend, the sequence, which can reflect the target chemistry signal to regulate the promoter strength, is currently tried to apply on the biosensor, which involved in the dynamic promoter regulation.

Another application of hybrid promoter strategy is to create the core regions of promoters. A new hybrid promoter was created by replacing TATA box and ATG region with a P_{PHO2} fragment and the part of core promoter P_{LEU2} (Madzak et al. 2000). Analogously to the TATA box assemble, the substitution of the conserved parts of the core promoters could be regarded as a special usage of hybrid promoter strategy in prokaryote. Guan et al. (2015) enhanced the expression of amino peptidase via P_{srfA} modification where the −10 and −35 core sequences were substituted with conserved sequences of σ^A-dependent promoters.

With the hybrid promoter strategy, the TFBSs can be utilized via tandem several upstream CRMs (UAS/URS). Gene expression could be increased monotonically with more TFBSs and mostly be saturated by 3 to 4 TFBSs (Fig. 2a) (Sharon and Al 2012). For instance, with three variants of Gal4p binding sites connected to the P_{LEUM} core promoters, a hybrid promoter library has been constructed that span a 50-fold range of controlled galactose-induced expression. The strongest hybrid promoter increased the transcriptional capacity of original promoter (Blazeck et al. 2012).

The strategy about tandemly repeat UAS (Fig. 2b) was confirmed to be effective for expanding the transcriptional capacity of promoter (Vinces et al. 2009). Blazeck et al. (2011) demonstrated that the activating regions and core regions can act as independent synthetic parts to create a hybrid promoter. They employed tandem UAS repeat elements to enhance the transcription strength of promoters for optimizing metabolic pathways.

The UAS elements from the mitotic cyclin *CLB2* gene and the mitochondrial citrate synthase *CIT1* gene were respectively cloned as UAS_{CLB} and UAS_{CIT}. The three copies of UAS_{CLB} or UAS_{CIT} sequences were fused at the upstream of core region of P_{LEUM}, P_{TEF}, P_{GPD}, and P_{CYC158} and it demonstrated that the transcription levels were all increased (Blazeck et al. 2012).

Meanwhile, the UAS from different sources, which means different kinds of TFBSs, play a vital role in promoter strength. Blazeck et al. (2014) increased itaconic acid titer to 59 mg/L from zero when the expression of *cis*-aconitic acid decarboxylase encoding gene (CAD) was driven by the UAS_{TEF}-UAS_{CIT}-UAS_{CLB}-P_{GPD} promoter. Different kinds of UAS elements and the order of these do influence the final strength of promoters. Shabbir-Hussain et al. (2017) determined that a 200-bp fatty acid responsive upstream regulatory sequence was suitable for combining URS in the P_{POX2} promoter based on the analysis of the promoter sequence with various multi-UASs and URSs hybrid tandem repeats. The hybridization resulted in a 48-fold increase in gene expression with glucose as inducer and 4-fold higher of production than the native P_{POX2} promoter. An alternative application of UAS and URS elements is to regulate the promoter strength and construct the libraries whose strength grads more trivial compared with the solo UAS/URS elements. Selecting and assembling the proper types and numbers of UAS have a great effect on transcription level. Previous researches demonstrated that shorter sequence length of USA leads to stronger transcription levels. The capacity of single UAS_{TEF} truncations elevated the strength of the P_{TEF} core promoter containing 136 bp to the 1.5-fold level of the native P_{TEF} promoter (Blazeck et al. 2013). Based on the one to twelve tandem repeats of the 230 nucleotide UAS fragments, the resulted hybrid promoter strengths were 3- to 4.5-fold to the original P_{TEF} promoter.

With the translation elements as hybrid materials, the hybridization of promoters and RBSs applied in various *E. coli* strains exhibited favorable protein expression level by similar application of hybridization strategy (Fig. 2c) (Gorochowski et al. 2014). The hybrid promoter strategy was adopted to synthesize 12,563 combinations of 114 common promoters and 111 RBSs under DNA, RNA, and protein levels from the entire library (Kosuri et al. 2013). Babiskin and Smolke (2011) inserted Rnt1p hairpin as hybrid elements within the 3′ UTR of a transcript to direct cleavage to that region. A library of synthetic control modules in yeast based on randomizing

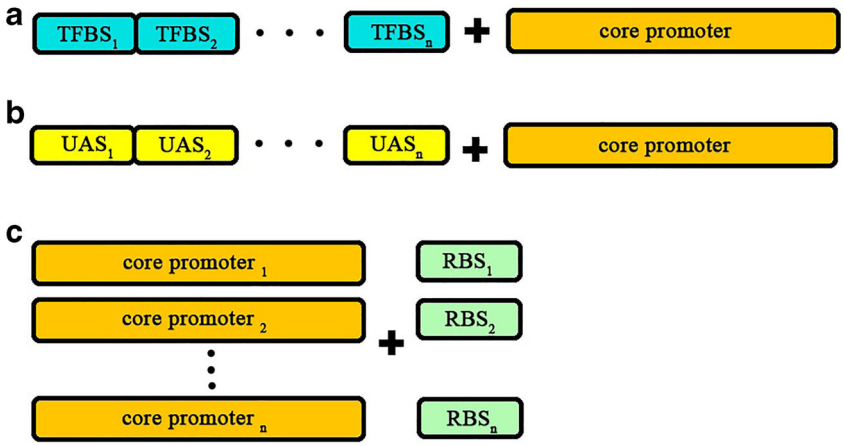

Fig. 2 The strategies of hybrid promoters. **a** Tandem TFBS hybrid to core promoter. **b** Tandem UAS elements hybrid to core promoter. **c** The hybrid strategy of promoters and RBS

the cleavage efficiency box was generated. As a result, these mutant hybrid elements of Rnt1p hairpins exhibited different gene regulatory activities through an in vivo screening. This new class of control elements can be combined with any promoter to support regulatory strategies encoded in transcriptional regulators and thus more sophisticated control schemes. Modification of the RBS and relative region regulates the ultimate expression level of the genes at the translation level, which broad the tuning range of gene expression.

The hybrid promoter strategy is a split-element strategy based on the desired functionality, which means that the requirements for the promoter structure are relatively low in this strategy. It was exampled that various transcriptional elements in this strategy have been tested to combine with the core region. The desired intensity enhancement, inducibility, and various other corresponding abilities can also be effectively altered in conferring new hybrid promoters through this strategy.

The application of promoter to balance multi-gene metabolism

Modularization balance of the metabolic pathway by promoters

Regulation of multi-gene co-expression by promoter engineering is usually applied in metabolic pathways, which could be realized by two strategies (Zhang et al. 2018a; 2018b): (i) modularization of the target-regulating genes according to their functions or pathways and (ii) dynamic regulation by cooperation of the multi-factors (Dunlop et al. 2010; Xu et al. 2014a; Zhang et al. 2012; Zhou et al. 2017b).

Under the control of single promoter, the natural gene clusters regulated the partial metabolic pathway uniformly as a kind of natural modularization. For the synthesis of 2,3-BD (2,3-Butanediaol), Xu et al. (2014b) studied the effects of promoters P_{abc}, P_c, P_{tac}, and P_{T7} on the expression of natural gene cluster *budABC*, and the result showed that the application of promoter P_{abc} was superior and 21.5 g/L of 2,3-BD was attained within 24 h. In view of the limited quantity of natural gene clusters, an artificial gene cluster could be constructed by gathering genes under the control of one promoter. For instance, Yim et al. (2011) sequentially cloned the genes *sucCD* (succinyl-CoA synthetase from *E. coli*), *sucD* (CoA-dependent succinate semialdehyde dehydrogenase), and *4hbd* (4-hydroxybutyrate dehydrogenase from *P. gingivalis*) to construct a gene cluster, which was driven by the PA1 promoter. As a result, the concentration of 4-hydroxybutyrate reached 11 mM. The strain BAK11 was constructed to produce salvianic acid by optimizing the three grouped modules of the metabolic pathway under different promoters. To eliminate the repression regulation of *LacI*, a 17.7-kb mao-paa cluster was replaced by artificial module 1 (P_{lacUV5}-*aroG*^{fbr}-*tyrA*^{fbr}-*aroE*) and the *lacI* gene was replaced by module 2 (P_{trc}-*glk-tktA-ppsA*) firstly. And the synthetic 5 *tac* promoter (stringing five repetitive *tac* promoters) provided the optimal strength to drive the expression of *hpaBC-d-ldh*^{Y52A} in module 3. As a result, the product yield reached 5.6 g/L after 60-h fermentation (Zhou et al. 2017a).

Gene clusters provide the natural unit for multi-gene expression, which reduced the energy dispersion of metabolism. However, there is also a limitation that the driving ability of promoters is influenced by the sequential order of genes and restrained by the unified regulation of a single promoter. To overcome these problems, the metabolic pathways are always divided into different modules based on similar function or nearby location of pathways. Shukal et al. (2019) firstly optimized transcription level of the engineered *Escherichia coli* to realize high production of 25.7 g/L viridiflorol by dividing the mevalonate pathway genes into two metabolic modules. In L-arginine metabolic pathway, the *opcA*, *pgl*, *tal*, *tkt*, and *zwf* genes in pentose phosphate pathway were sorted out as an NADPH level regulator to increase the production of L-arginine (Park et al. 2014). This module was overexpressed by a strong promoter P_{sod}, resulting in a NADPH level of

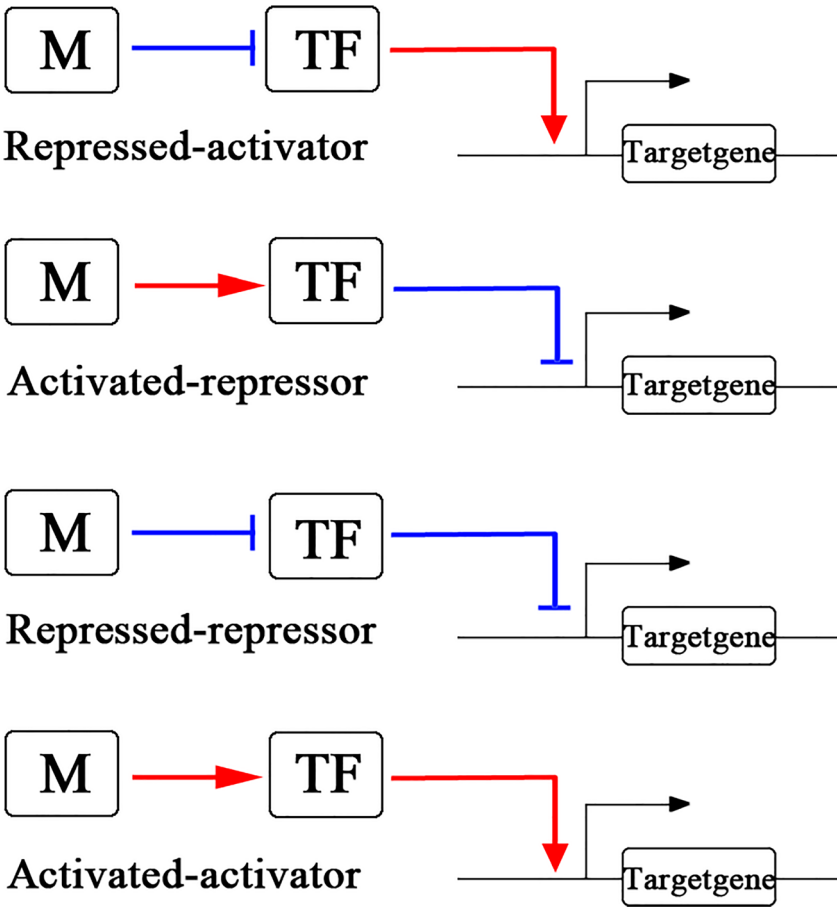

Fig. 3 Metabolites (M) respond to the TFs and TFs binding to promoters

82.6 mmol g DCW^{-1} in *C. glutamicum*. In addition, five graded strength-descending promoters, P_{TDH3}, P_{TEF1}, P_{RPL18B}, P_{RNR2}, and P_{REV1}, were selected to simulate the graded promoter-levels in promoter libraries. Matsushika et al. (2009) divided the main xylose converting pathway to two modules; three genes (xylose reductase, xylitol dehydrogenase, and xylulokinase) were sorted out to make the xylitol metabolic access as *the partial way* and the pentose phosphate pathway that contains eight genes (expanded with non-oxidative pentose phosphate pathway enzymes and pyruvate kinase) as *the full way*. The interplay between the upstream xylose utilization pathway and the downstream pentose phosphate pathway was investigated by comparing the enrichment profiles in different genetic contexts and the enzymes responsible for the production bottleneck were confirmed (Latimer et al. 2014). Multi-gene sort mitigates, to a certain extent, the discrepancy among the genes in the same module while the contradictions between different modules require proper promoters to harmonize.

Above cases well-illustrated how to wipe off the useless genes in modules to precisely modify the key genes and pick up the matched strength promoters to optimize the production by the promoter replacement strategy. It could be generalized that the application of the graded strength promoters can regulate the metabolic balance well through the simulation and speculation of metabolic pathway.

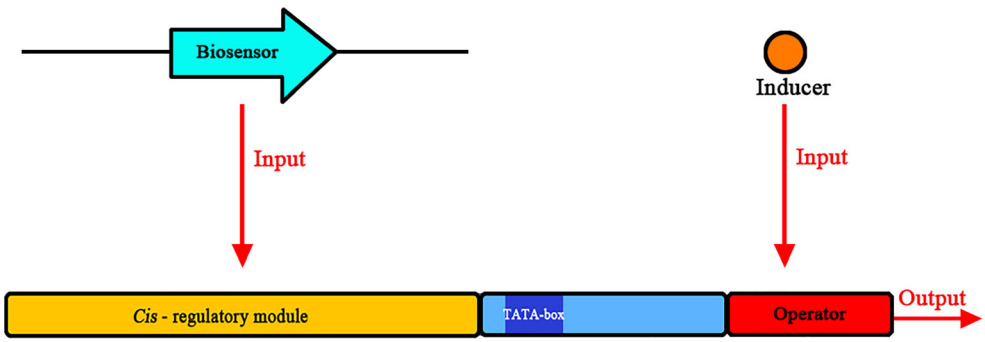

Fig. 4 AND—logic gate compose of the biosensor and the operator

Table 1 The list of promoter and metabolic strategies

Section	Strategy	Product/Result	References
Site-selective mutagenesis strategy	A model of sequence-dependent binding energy calculation combined with a thermodynamic model of transcription	Higher the target gene level	Brewster et al. 2012
	An in *silico* analysis to construct a deletion library	Methanol	Hartner et al. 2008
	Energy matrix to design a *trc* promoter library	Succinate production	Kinney et al. 2010; Yu et al. 2016
Hybrid promoter strategy	Tandem TFBSs	A 50-fold range of hybrid promoter library	Blazeck et al. 2012
	Tandem multi-URS	Itaconic acid	Blazeck et al. 2014
	Conserved sequence replacement	A new hybrid promoter	Guan et al. 2015
	Hybridization with operon	Developed a new cumate (p-isopropylbenzoic acid)-inducible gene switch	Horbal et al. 2014
	Hybridization with operon	Surfactin	Jiao et al. 2017
	Hybridization of tata box and atg region	A new hybrid promoter	Madzak et al. 2000
Modularization balance of pathway by promoters	Divide the metabolic network into various promoter controlled modules	Xylitol	Latimer et al. 2014; Matsushika et al. 2009
	Divide the mevalonate pathway genes into two metabolic modules	Viridiflorol	Shukal et al. 2019
	Probable promoter controlled the gene cluster	2,3-Butanediaol	Xu et al. 2014b
	Probable promoter controlled the gene cluster	4-Hydroxybutyrate	Yim et al. 2011
	Divide the metabolic network into various promoter controlled modules	Salvianic acid	Zhou et al. 2017a
Dynamic balancing of pathway by promoters	Auto-regulated transcription factor to construct a dynamic sensor-regulator	3,4-Dihydroxy benzoate	Jha et al. 2014
	Logic circuits to combine various dynamic control strategies	Acetate	Moser et al. 2018
	Logic circuits to combine various dynamic control strategies	And-gate regulated system	Teo and Chang. 2013
	Auto-regulated transcription factor to construct a dynamic sensor-regulator	Fatty acid	Xu et al. 2014a
	Auto-regulated transcription factor to construct a dynamic sensor-regulator	Biodiesel	Zhang et al. 2012

Dynamic balancing of the metabolic pathway by promoters

To screen libraries with high genetic diversity, the key problem is the screening or selection technique with high throughput. Microbial biosensor linking together an enzyme-catalyzed product with a transcription factor that recognizes the biocatalytic product can perform high-throughput selection. The dynamical regulation of the metabolic pathways is available based on the combination of metabolic feedback analysis to TFs and TFs binding with the promoters. The endogenic expression of TFs exhibited advantage of auto-regulation for the metabolic balance. The identification and application of other DNA binders that respond to chemical ligands or to other signals (Ramos et al. 2005) can enable the construction of more synthetic regulatory networks (Fig. 3). Biosensor as a kind of dynamic regulator of metabolite has been studied widely. The way of constructing a biosensor in bacteria is to introduce an endogenous inducer as TFs and assemble it with the corresponsive promoters. An auto-regulated transcription factor, pcaU, was cloned from *Acinetobacter* sp. ADP1 to *E. coli* and its promoter region adapted for activity in *E. coli*. By inputting an inducible plasmid that promoted the metabolism of 3,4 dihydroxy benzoate (34DHB), a sensitive biosensor can be generated since the engineered transcription factor pcaU regulon was inducible at 34DHB (Jha et al. 2014).

Fatty acid (FA) is a common signal to feedback the transcription factor in biosensor. For instance, a fatty acid/acyl-CoA (FA/acyl-CoA) biosensor based on the fatty acid-sensing protein FadR has been developed. FadR is a ligand-responsive transcription factor that binds to specific DNA sequences and controls the expression of several genes involved in fatty acid (FA) biosynthesis (Yasutaro et al. 2010). When FA was present, acyl-CoA synthase was activated. Acyl-CoA in turn binded to FadR and FadR was released from the synthetic promoter, initiating the target gene transcription. The binding sequence was hybridized into non-conserved spacers of a phage lambda promoter (P_L) and a phage T7 promoter (P_{A1}), respectively, to design two synthetic

FA/acyl-CoA-regulated promoters. As a result, this dynamic sensor-regulator system improved the stability of biodiesel-producing strains and increased the biodiesel titer to 1.5 g/L (Zhang et al. 2012). FapR was a transcription repressor that specifically senses malonyl-CoA and regulates gene expression in the *B. subtilis* FA biosynthetic pathway. Xu et al. (2014a) found that TF FapR had a comparable binding affinity toward UAS of P_{GAP}. By incorporating TF FapR and UAS of P_{GAP}, a malonyl-CoA sensor has been identified, which exhibited malonyl-CoA-dependent transcriptional activity. Implementation of this metabolic control resulted in an oscillatory malonyl-CoA pattern and a balanced metabolism between cell growth and product formation, yielding a significant improvement in FA titer compared with the wild-type strain.

The logic circuits (Singh 2014), based on operon and biosensor as signal inputs, were constructed by cooperation of multi-dynamic controllers (Fig. 4). Teo and Chang (2013) customized AND-gate dynamic controllers in *S. cerevisiae* by combining inducible promoters and sensing-regulation together. With the fatty acid responsive FadR repressor and its operator sequence, the AND-gate dynamic controllers were established to switch the AND-gate ON. Moser et al. (2018) designed glucose, oxygen, and acetate sensors by respectively binding the operators to cAMP receptor protein (CRP), FNR transcriptional activator, and the nitrogen regulator protein I (NRI or NtrC) (Bulter et al. 2004). To control *pta* (the primary route to acetate production), a circuit was developed that responds in the presence of glucose under certain oxygen. The corresponding strain greatly reduced acetate accumulation during exponential growth, yielding a 4-fold reduction in acetate after 27-h growth compared with the control (Moser et al. 2018). Logic circuits provide multiple conditional regulations of cellular metabolism, effectively increasing the accuracy requirements for gene expression and reducing the effect of infinitesimal inducers on transcription.

Perspective

Promoter engineering currently relies primarily on random mutation or rational design to create libraries, providing a large number of samples for further modification. Promoters strategies (Table 1) included mathematical construction methods based on the promoter structural characteristics and hybrid promoter strategy based on the function of the elements, which provided the basis for dynamic regulation of metabolic engineering with high efficiency. The dynamic regulation of the promoter, especially the application of biosensors, made the metabolic pathways more straightforward. The logic gate regulation strategy was enriched by the regulation elements of dynamic regulation, which realized the dynamic control of metabolic balance by multiple factors.

Promoter engineering mainly controls the metabolic network by regulating the transcription level. In addition, combining translation levels and post-translational modifications to improve the optimization of the entire enzyme expression process can further enhance the precision of gene expression levels and increase the production of metabolites. Especially for the great potential of TFs, transcription factor engineering has not only been limited in proteins-DNA, but also extended to proteins-proteins. Exploring more types of transcription factors (such as zinc-finger protein transcription factors) and the development of external transcription factors are conducive to precisely control the metabolic balance.

Funding information This work was funded by National Natural Science Foundation of China (Grant No. 21602199).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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