Title

Fine-tuning gene expression for improved biosynthesis of natural products: From transcriptional to post-translational regulation.

Authors

Jiang, Tian
Li, Michelle
Zou, Yusong
et al.

Publication Date

2022

DOI

10.1016/j.biotechadv.2021.107853

Peer reviewed

HHS Public Access

Author manuscript

Biotechnol Adv. Author manuscript; available in PMC 2023 January 01.

Published in final edited form as:

Biotechnol Adv. 2022 ; 54: 107853. doi:10.1016/j.biotechadv.2021.107853.

Fine-Tuning Gene Expression for Improved Biosynthesis of Natural Products: From Transcriptional to Post-Translational Regulation

Chenyi Li[1], **Tian Jiang**[1], **Michelle Li**[2], **Yusong Zou**[1], **Yajun Yan**[1,*]

[1]School of Chemical, Materials and Biomedical Engineering, College of Engineering, The University of Georgia, Athens, GA, 30602, USA

[2]North Oconee High School, Bogart, GA 30622, USA

Abstract

Microbial production of natural compounds has attracted extensive attention due to their high value in pharmaceutical, cosmetic, and food industries. Constructing efficient microbial cell factories for biosynthesis of natural products requires the fine-tuning of gene expressions to minimize the accumulation of toxic metabolites, reduce the competition between cell growth and product generation, as well as achieve the balance of redox or co-factors. In this review, we focus on recent advances in fine-tuning gene expression at the DNA, RNA, and protein levels to improve the microbial biosynthesis of natural products. Commonly used regulatory toolsets in each level are discussed, and perspectives for future direction in this area are provided.

Keywords

Fine-tuning gene expression; Natural products; Dynamic regulation

1. Introduction

Natural products, such as terpenoids, flavonoids, and alkaloids, are wildly used as nutritionally or pharmaceutically important components in biomedicine applications, dietary supplements, and cosmetic industries (Chen et al., 2015; Kusumawati and Indrayanto, 2013; Liu and Nielsen, 2019; Rodrigues et al., 2016; Ververidis et al., 2007). Recent years have witnessed a rapidly growing demand for natural products (Wang, R. et al., 2020; Yang et al., 2020). This high demand motivates the exploration into cost-effective manufacturing

*Correspondence: yajunyan@uga.edu.

Author contributions

C.L. drafted the manuscript. T.J., M.L., and Y.Y. revised the manuscript. Y.Z. assisted in figure creation. All authors reviewed and approved the manuscript.

Conflict of Interest

The authors declare no competing interest.

of natural products. In recent years, to improve the production capacity, constructing microbial cell factories for these value-added compounds has drawn a lot of attention. The past decades have witnessed rapid development in metabolic engineering of microbial hosts for the production of natural compounds (Cravens et al., 2019; Zha et al., 2020; Zhang et al., 2018). With the expanding genetic toolsets, some amenable microbial hosts with clear genetic backgrounds and strong production capacities were explored for natural product synthesis, including *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae,* and *Corynebacterium glutamicum* (Calero and Nikel, 2019; Fletcher et al., 2016). Fast dividing microorganisms (0.5–3 h doubling time) can enable shortened production cycles. The purification workflow can also be potentially simplified since only a limited range of similar metabolites are endogenously produced in commonly used industrial microbial hosts (Ehrenworth and Peralta-Yahya, 2017). In addition to the aforementioned benefits, the ease of cultivation and manipulation, as well as potentially lower production costs, have attracted more attention in developing efficient microbial cell factories for biosynthesis of natural products (Ehrenworth and Peralta-Yahya, 2017; Wang et al., 2018). Thus, exciting achievements have been made in the microbial production of small aromatic compounds (Chouhan et al., 2017; Liu, Q. et al., 2019; Wang et al., 2018), terpenoids (Ajikumar et al., 2010; Keasling, 2010), flavonoids (Biggs et al., 2016; Liu, X. et al., 2018; Palmer et al., 2019), and polyketides (Wang, W. et al., 2020; Weissman, 2016) in the past decades.

Despite the advantages of utilizing microbial hosts to synthesize natural products, there are many challenges in constructing efficient microbial cell factories for producing these value-added compounds. First, the production of many natural compounds requires the consumption of precursors from microbial central metabolism. The synthesis of these compounds can cause competition between cell growth and production, which may negatively affect the productivities of microbial chassis (Wu et al., 2016). Additionally, the biosynthetic pathways for natural products usually involve metabolites that do not naturally exist in the host, and the accumulation of these chemicals can be toxic to the chassis and thus hinder the production efficiencies (Jiang et al., 2020). Moreover, some reactions in the synthesis of natural compounds require the input of co-factors or redox molecules. The consumption of these biomolecules may cause imbalances of co-factors, which can also hamper cell growth and production.

To address these problems, a series of metabolic engineering strategies, including adaptive laboratory evolution (Sandberg et al., 2019), protein/enzyme engineering (Li et al., 2020), compartmentalization (Abernathy et al., 2017; Hammer and Avalos, 2017), scaffold engineering (Park et al., 2018), co-factor engineering (Black et al., 2020; Zhao, X. et al., 2015), dynamic pathway regulations (Chen and Liu, 2018; Jones, J. A. et al., 2015; Shen et al., 2019), and combinatorial optimizations (Galanie et al., 2015; Jeschek et al., 2016; Zhao, S. et al., 2015), have been widely applied to improve the biosynthesis of natural products. Besides these strategies, fine-tuning the gene expression, either via controlling the biosynthetic pathways of natural products or regulating the central metabolisms of the microbial chassis, has been proven to be a promising and efficient way to address these challenges. When the microbial cell factories are reasonably regulated, the engineered strains can achieve better cell growth and higher titers. Gene expression can be typically controlled at three levels: the DNA level, (transcriptional level), RNA

level (post-transcriptional and translational level), protein level (post-translational level), and multilevel regulation. In this review, we focus on recent advances in fine-tuning gene expression at these three levels to improve the biosynthesis of natural products. Commonly used toolsets in each level are reviewed and outlooks for future direction in this area are provided.

2. Regulating the biosynthesis of natural products

Due to the stochasticity and complex regulations lying in microbial systems, sometimes it is challenging to achieve high productivities of natural products in microbial hosts (Kaern et al., 2005; Rugbjerg et al., 2018). The synthetic pathways for natural products usually involve multiple steps, and some metabolites may be harmful to microbial hosts (Dahl et al., 2013; Yang et al., 2020). Therefore, controlling the gene expression to reduce the competition between cell growth and production, avoid or delay the accumulation of toxic metabolites, and alleviate the feedback inhibition, is necessary for achieving high titers of value-added natural compounds. Plenty of efficient regulation toolboxes or novel regulation strategies have been established and well-characterized in recent years, which greatly promoted the fine-tuning of gene expression in natural product biosynthesis (Table 1). These strategies were divided into three levels and discussed in this paper based on which process they regulate.

2.1 Regulation at DNA level

The first step in protein expression is the transcription of DNA to RNA, where the promoters, RNA polymerase, sigma factors, and allosteric transcriptional factors (aTF) are typically involved (Clancy, 2008). Plenty of transcriptional regulation strategies and toolsets have been developed in the past decades, such as promoter engineering (Jones, J. Andrew et al., 2015; Lim Chin et al., 2011; Zhou et al., 2019), allosteric transcriptional factors (Mahr and Frunzke, 2016), CRISPR-Cas based gene regulation (Gilbert et al., 2014), TALE-mediated (transcriptional activator-like effector) transcriptional control (Crocker and Stern, 2013), zinc finger-mediated transcriptional control (Laity et al., 2001), and DNA aptamers (Jang et al., 2017; Wang et al., 2017). Regulation at the DNA level is to control the transcription of target genes, which is the most common strategy in pathway control due to the ease of manipulation. As transcription is the start of protein synthesis, inhibition on transcription would also negatively affect the translation and post-translation modifications. Hence, when applying DNA level regulation, the whole protein synthesis process can be regulated with just a simple control.

Promoters directly affect the transcription rate, and thus are common targets used for engineering biosynthesis of natural products (Alper et al., 2005; Kang et al., 2016). Constructing promoter variants with different transcriptional activities and applying them to fine tune the biosynthetic pathways are becoming increasingly feasible with the development of biosensors and high-throughput screening techniques (Lee et al., 2013; Xiu et al., 2017; Zhou et al., 2019) . Naringenin is a valuable natural product with great therapeutic potential (Salehi et al., 2019). The microbial synthesis of naringenin is achieved via converting the tyrosine to *p*-coumaric acid and then *p*-coumaroyl-CoA by TAL and 4CL, followed by

condensation with three molecules of malonyl-CoA and isomerization to form naringenin by CHS and CHI (Miyahisa et al., 2006). These four genes need to be fine-tuned to balance the carbon flux so that the naringenin production can be enhanced. Therefore, an iterative high-throughput balancing (IHTB) strategy was established to thoroughly fine-tune the naringenin biosynthetic pathway (Zhou et al., 2019). Constitutive promoters with gradient strengths were randomly picked to control the expression of pathway genes, resulting a library with differed naringenin production capacities. After screening of over 1,200 candidates, the metabolic flux of the naringenin synthetic pathway was appropriately balanced, with the final naringenin titer reaching 191.9 mg/L (Zhou et al., 2019). Similar strategy has also been demonstrated in engineering the naringenin production in *S.cerevisiae* (Wang, R. et al., 2019). Promoter engineering is a common strategy to fine-tune the gene expression at DNA level, but the large library size usually make the characterization processes rely on the high-throughput screening techniques (Lee et al., 2013). Thus, future engineering and development of efficient high-throughput detection techniques would benefit and accelerate the processes of developing high-producers using promoter engineering.

Allosteric transcriptional factor (aTF) is the most commonly used tool in controlling gene expression at the DNA level (Latchman, 1993). aTF is a regulatory protein that can control, either by enhancing (transcriptional activator) or inhibiting (transcriptional repressor), the transcription by binding to a specific DNA sequence in the promoter region (Fig. 1a). This region often overlaps with the −35 or −10 box that is for RNA polymerase binding (Karin, 1990; Shen et al., 2019). Due to the ligand-sensing ability of aTFs, they were usually used to enable dynamic and real-time fine-tuning of the gene expression for natural product synthesis. The first dynamic control was achieved in lycopene biosynthesis by Liao's group (Farmer and Liao, 2000). After that, this strategy was wildly applied in the synthesis of isoprenoid, fatty acids, glucaric acid, flavonoids, and aromatic compounds (Dahl et al., 2013; Dinh and Prather, 2019; Doong et al., 2018; Gupta et al., 2017; Liang et al., 2020; Xu et al., 2014; Yang et al., 2018). The regulatory circuits in these studies are powered by the inducible aTFs to improve the production of the target compounds and reduce the competition between cell growth and production. For example, vanillin is a valuable natural product with a projected market size of USD 734.5 million in 2025 (Global Vanillin Market Size). Despite the high value of this compound, the microbial synthesis of vanillin can compete with cell growth, and high pathway expression can lead to growth defects and plummeted productivity as accumulated vanillin displays antimicrobial activity (Liang et al., 2020). To solve these problems, a dynamic pathway control circuit powered by the aTF HucR variants was designed and applied to vanillin biosynthesis. The delayed pathway expression significantly improved the cell growth, which in turn resulted in an increased vanillin production to a titer of over 1.5 g/L (Liang et al., 2020). Plant-derived synthetic aTFs were implemented in improving the production of β-ionone and naringenin in *Saccharomyces cerevisiae* (Naseri et al., 2019). Through balancing the pathway expression, a 4.2-fold increase in β-ionone production was achieved. The improved production of naringenin was also detected by a biosensor (Naseri et al., 2019). While the aTF is a powerful tool to regulate gene expression, the limited substrate scopes and narrow dynamic ranges of natural aTFs may constrain their application potentials in dynamic gene regulations. Mining new aTFs or engineering existing aTFs to respond to new compounds

and improving the dynamic range of existing aTFs (Jiang et al., 2021) are becoming increasingly important to expand the applicability of aTF in fine-tuning natural product biosynthesis (Li et al., 2020).

In addition to the aTFs and promoter engineering, CRISPR (clusters of regularly interspaced short palindromic repeats) based gene regulation is also a trending tool for improving the natural product biosynthesis (Fig. 1b). By using the nuclease-deficient CRISPR protein (dCas9), transcriptional activation (CRISPRa) (Bester et al., 2018; Fontana et al., 2020; Konermann et al., 2015) and interference (CRISPRi) (Gilbert et al., 2014; Wu et al., 2020; Zhao et al., 2021) have been demonstrated in various hosts for efficient transcriptional control (Lian et al., 2019). An important advantage of using CRISPR-based gene expression control is the ease of achieving simultaneous multiplex regulation. For example, the biosynthesis of naringenin requires the input of malonyl-CoA and *p*-coumaric acid derived from tyrosine. Thus, CRISPRi was applied in fine-tuning multiple target genes involved in the central metabolism of *E. coli* to rewire the carbon flux to the precursor of naringenin (Wu et al., 2015). The simultaneous inhibition of *fabF, fumC, fabB, sucC,* and *adhE* resulted in a 7.6-fold increase in naringenin titer to 421.6 mg/L without significantly altering the final biomass accumulation (Wu et al., 2015). In another study, the simultaneous down-regulation of multiple competing pathways using CRISPRi combined with fermentation optimization enhanced the production of β-amyrin in *S. cerevisiae* to 156.7 mg/L, a 44.3% increase in the titer compared with the unregulated strain (Ni et al., 2019). CRISPRi based control was also employed to alleviate the feedback inhibition lying in the biosynthetic pathway (Sander et al., 2019). Amino acid biosynthesis is known to be tightly regulated in microbial cells. Sander and colleagues applied CRISPRi in downregulating the feedback inhibition mediated by ArgR in *E. coli* and improved the cell growth and production of the arginine-overproducing strain (Sander et al., 2019). As for CRISPRa, this system has been well-established in engineering the biosynthesis of natural products such as violacein (Liu, Y. et al., 2019) and epothilone (Fontana et al., 2020; Peng, R. et al., 2018). In 2021, CRISPRa was ported and engineered to function in *Pseudomonas putida* (Kiattisewee et al., 2021). With systematic analysis and optimizations, CRISPRa was applied to regulate the biosynthesis of biopterin and mevalonate, resulting in over 5-fold and 40-fold increase in titers, respectively (Kiattisewee et al., 2021). Besides the application of simple inhibition or activation, CRISPR-based multiplex regulation for the production of β-carotene has also been demonstrated in yeast (Lian et al., 2017). By up-regulating the rate-limiting step catalyzed by *HMG1*, inhibiting the *ERG9* that competes the β-carotene biosynthesis for endogenous sterol generation, and deleting a stress responsive transcriptional regulator *ROX1*, the titer of β-carotene increased by up to 2.8-fold compared to the control strain (Lian et al., 2017). As a trending regulation method, CRISPR-based gene expression control demonstrated great potentials in improving the biosynthesis of natural products. Future optimization of the CRISPR system such as minimizing the off-target effect and engineering relaxed PAM specificity may continue to drive the application of this regulation method in metabolic engineering (Chatterjee et al., 2020; Collias and Beisel, 2021).

DNA aptamers that can bind to specific ligands are also used to construct genetic circuits which control the gene expression at the transcriptional level (Fig. 1c) (Pfeiffer and Mayer, 2016; Ruscito and DeRosa, 2016). The binding of ligand would cause unwinding of double

stranded DNA, which would allow easier recognition and binding by RNA polymerases if the aptamer is placed near the promoter region, leading to the enhanced expression of downstream genes at the transcriptional level (Wang et al., 2017) (Fig. 1c). The well-characterized thrombin-bound aptamer was engineered and applied into 2′-fucosyllactose synthesis in *B. subilits* (Deng et al., 2019). The introduction of DNA aptamer-mediated control enabled a titer of 511 mg/L, a 22.3-fold increase over the parental strain (Deng et al., 2019). While the regulation mediated by DNA aptamers is easy to achieve, the limited set of available DNA aptamers significantly hindered the further applications of this regulatory component. Therefore, larger libraries of signal molecules and responsive components are required in the future for multiplex, fine-tuning natural product biosynthesis via DNA aptamers.

While not as common as other regulation tools at DNA level, the TALE-mediated regulation was also applied in engineering biosynthesis of natural product. In 2016, TALE-fused enzymes were gathered around the TALE DNA scaffolds and enriched the local enzyme concentrations, which led to a 9.6-fold increase in inole-3-acetic acid production (Zhu et al., 2016).

Applying transcriptional regulation strategies for tunable gene expression at DNA level, which is the start of protein expression, can control the synthesis of target proteins with minimal effort. Thus, it is widely used in metabolic pathway control, especially for natural products. While this type of regulation is efficient and easy to achieve, the stop of transcription did not necessarily represent a decreased availability of proteins, because the previously transcribed mRNA can still be translated to proteins by the ribosomes, and there are also residual enzymes/proteins in the cells that can still function as normal (Sekar et al., 2016; Yuan and Ching, 2015). Therefore, if only the transcriptional control is applied, there will often be a time lag for the cells to exhibit the regulation effects due to the above reasons, and this type of control sometimes can be less effective in some microorganisms if the vast majority of intracellular proteins are long-lived, such as *S. cerevisiae* and *S. pombe* (Christiano et al., 2014; Yuan and Ching, 2015).

2.2 Regulation at RNA level

Messenger RNA (mRNA) is the bridge connecting DNA and proteins. RNA-level regulations are usually achieved by blocking the access of ribosome to the ribozyme binding site (RBS) in mRNA or decreasing the mRNA availabilities to hinder the translation and thus decrease the protein expression level. The commonly used tools include small regulatory RNAs, antisense RNAs, and riboswitches (Wang and Cirino, 2016).

Small regulatory RNA (sRNA) is a type of short non-coding RNA that natively exists in many microorganisms (Fig. 2a). It was first discovered in *E. coli* in 1984 (Mizuno et al., 1984; Svensson and Sharma, 2016; Vogel and Wagner, 2007). sRNA consists of two major components: one is the scaffold that is used to stabilize the RNA structure, the other is a short binding sequence (usually 20-200 bp) that can bind with target mRNA by complementary pairing. When the target region includes the RBS, the binding of sRNA can both block the access of RBS and also accelerate the degradation of mRNA to decrease the mRNA concentration. When the RBS is not in the sRNA target region,

the binding of sRNA would only cause a decreased availability of mRNA (Aiba, 2007; Culver, 2001). Notably, the degradation of the RNA complex upon binding with target mRNA requires the recruitment of hfq protein, an abundant bacterial RNA binding protein that is physiologically important in interacting with sRNAs and facilitating their antisense interactions with their targets (Holmqvist et al., 2016; Tsui et al., 1994). In 2013, Sang Yup Lee and coworkers established a standard workflow to engineer the small regulatory RNA (sRNA) for regulating biosynthesis pathways (Na et al., 2013; Yoo et al., 2013). By screening over 100 candidate sRNAs, they select the micC as the optimal scaffold to design and engineer synthetic sRNAs. The designed sRNAs were applied to inhibit the competing pathways of lysine biosynthesis and rewire the carbon flux from central metabolism to cadaverine production. They screened a library containing 130 synthetic sRNAs, and a 55% increase in cadaverine titer was achieved when repressing *murE* (Na et al., 2013). In 2019, the same group developed an expanded synthetic sRNA expression platform to enable rapid, multiplexed, and genome-scale target gene knockdown by improving its compatibility for commonly used antibiotic markers and origin of replications (ORI, e.g. p15A, ColE1, and pSC101) (Yang et al., 2019). Using this strategy, they demonstrated increased productions of L-threonine (22.9 g/L), L-proline (54.1 g/L), crude violacein (5.19 g/L), and indigo (135 mg/L) with corresponding engineered strains that were developed from other studies (Yang et al., 2019).

Antisense RNA (asRNA) is also a common tool used for RNA-level regulation (Fig. 2b). The function of asRNA is very similar to sRNA: the RNA-level regulation mediated by asRNA is also achieved by complementary pairing with target mRNA, but unlike sRNA, the degradation of the asRNA-mRNA complex does not require the recruitment of hfq protein (Pelechano and Steinmetz, 2013). Instead, it is usually achieved by the native RNA degradation enzymes, such as RNAse III in Gram-positive bacteria (Lasa et al., 2011). Most notable applications of asRNAs are in improving the biosynthesis of flavonoids. In 2015, the antisense RNA was engineered to rewire carbon flux from central metabolisms to flavonoids biosynthesis (Wu et al., 2014; Yang et al., 2015). The asRNA was equipped with a 100 bp artificial stem loop in these two studies to improve its stability. The fatty acid synthesis pathway was inhibited by the engineered asRNA to enhance the availability of malonyl-CoA, an important precursor for the synthesis of a series of flavonoids. In Yang's paper, the asRNA-mediated down-regulation was applied towards fabD, which converts the malonyl-CoA to fatty acids. This regulation resulted in a 4.5-fold increase of the malonyl-CoA availability, which in turn enabled a 1.53-fold, 1.70-fold, and 2.53-fold increase in the production of naringenin, resveratrol, and 4-hydroxycoumarin, respectively (Yang et al., 2015). In Wu's paper, the inhibition of fabB/fabF using asRNA improved the production titer of naringenin by 4.31-fold (391 mg/liter) (Wu et al., 2014). These examples demonstrated that asRNA is a powerful tool for fine-tuning the central metabolism of bacteria to improve the biosynthesis of natural products.

Unlike the sRNA and asRNA, regulation mediated by riboswitch does not affect the intracellular mRNA availability. Riboswitch is a type of regulator that controls the gene expression via conformational change of RNA secondary structures (Fig. 2c) (Mandal et al., 2003). There are two major components in a typical riboswitch, one is the ligand binding domain, where the binding of target compounds can cause a conformational change to

the secondary structure. The other is the regulation domain, where the RNA secondary structure can block the access of ribozyme to RBS and thus hinder the translation. While this type of regulator is wildly distributed in microorganisms, they were often used as biosensors to screen high-producers (Lee and Oh, 2015; Wang et al., 2015; Yang et al., 2013), and only a handful of examples have achieved gene regulation via riboswitches. Deng and co-workers achieved simultaneous up-regulation and down-regulation of *in vivo* gene expression by engineering the ligand thrombin-bound aptamer (Deng et al., 2019). Using this, they enhanced the expression of 2′-fucosyllactose pathway genes but reduced the unnecessary induce of biofilm formation in *Bacillus subtilis*. The final strain reached a titer of 674 mg/L when the bi-functional regulation was applied, which was 27.3-fold that of the un-controlled original strain (Deng et al., 2019). This type of dual-control has also been demonstrated in another study. The lysine-responsive riboswitches were applied in *Corynebacterium glutamicum* for metabolic control (Zhou and Zeng, 2015). The screened lysine-ON riboswitches were used to control the expression of *lysE* gene (encoding a lysine transport protein), achieving dynamic up-regulation of lysine transport in the *C. glutamicum.* On the other hand, a lysine-OFF riboswitch was applied to dynamically down-regulate the expression of citrate synthase. The combined dual-control enabled an 89% increase in lysine yield, and also demonstrated the synergetic effect of lysine-ON and -OFF riboswitches for improving lysine production (Zhou and Zeng, 2015).

Instead of using regulatory proteins compared with transcriptional regulation, the RNA-level regulations are usually achieved via non-coding RNAs. Introducing such non-coding RNAs can often bring less cell burden to the host than using regulatory proteins. First, non-coding RNAs (usually less than 200 nt) are often shorter than normal mRNA (usually over 200 nt). Moreover, compared with proteins that need to be synthesized from amino acids, RNAs do not consume amino acids for synthesis, which can effectively save some resources for cell growth or production. However, similar to DNA-level control, even though the RNA-level regulation can decrease the availability of mRNA or hinder the translation, this cannot quickly transition to a decreased protein level, because the residual proteins in cells usually would not be quickly degraded so they can still function as normal (Sekar et al., 2016; Yuan and Ching, 2015). Also, when applying RNA-level regulation, the synthesis of mRNA (transcription) is not controlled, which means the mRNA can still be synthesized continuously, which can cause a waste of cell resources for unnecessary synthesis of mRNAs.

2.3 Regulation at protein level

One of the essential goals for gene regulation is to adjust the intracellular protein concentrations. Therefore, regulation at the protein level provides a more direct way to achieve fast and accurate control of the protein availabilities and thus has attracted increasing attention in recent years (Cameron and Collins, 2014; Chung et al., 2015). Unlike the regulations at DNA or RNA level that can achieve both up- and down-regulation (Lian et al., 2017; Zhou and Zeng, 2015), most studies with protein level control usually focused on controllable degradation (down-regulation). Down-regulation by tunable protein degradation reduces the intracellular protein availabilities to a low level, which would be helpful in shutting down competing pathways or rewiring carbon fluxes. The major ways to achieve

tunable protein degradation are through engineering protein degradation tags or degrons, as well as controllable expression of proteases (Fig. 3) (Cameron and Collins, 2014; Gao et al., 2019; Lu et al., 2021).

Regulated protein depletion via degradation tags or degrons was commonly used in terpenoids biosynthesis (Lu et al., 2021; Peng, B. et al., 2018; Peng et al., 2017). Terpenoids biosynthesis requires the consumption of building blocks like isopentenyl pyrophosphate (IPP), geranyl pyrophosphate (GPP), and farnesyl pyrophosphate (FPP), but these precursors are also essential for biomass generation and cell growth. Therefore, the carbon flux needs to be appropriately controlled to minimize the competition of these precursors between cell growth and production. To this end, an autonomous controllable degradation was established in *S. cerevisiae* via a PEST (rich in Pro, Glu/Asp, Ser, and Thr) sequence-dependent endoplasmic reticulum-associated protein degradation (ERAD) mechanism (Peng et al., 2017). The existence of Erg9p would direct excessive FPP to sterol synthesis and decrease the production of the target product nerolidol, but transcriptional inhibition of Erg9p was insufficient to regulate its availability due to the high stability of the enzyme (Christiano et al., 2014; Yuan and Ching, 2015). As a result, the protein-level regulation via the degradation tags was carried out to control the intracellular Erg9p availability. The Erg9p protein can be normally expressed and function during the growth phase, but it will be degraded during the production phase. Through this strategy, they successfully minimized the competition between cell growth and nerolidol production and enabled an 86% increase in the final titer (Peng et al., 2017). Inspired by this achievement, the same group further applied a similar strategy towards controlling the endogenous farnesyl pyrophosphate synthase (FPPS, Erg20p) to minimize the competition between biomass generation and linalool production, with a 27-fold increase in the final titer (Peng, B. et al., 2018). Recently, an engineered auxin-inducible protein degradation strategy for several terpenoids biosynthesis was developed (Lu et al., 2021). The natural auxin-inducible protein degradation system suffers from auxin-independent basal degradation, making it troublesome for controlling specific responses (Li et al., 2019; Nishimura et al., 2009). Hence, in the referred study, the auxin-inducible degradation system was optimized and improved with reduced non-auxin-dependent degradation. The engineered system was applied to improve the production of limonene and nerolidol. The titer of the monoterpene limonene and sesquiterpene (C15) nerolidol was increased to 76 mg/L and 3.5 g/L in flask cultivation, respectively (Lu et al., 2021).

While most studies focus on the employment of degradation tags and degrons, Cong and colleagues established a dynamic protein degradation genetic circuit via biosensor-controlled protease expression (Gao et al., 2019). Three orthogonal proteases, tobacco etch virus protease (TEVp), tobacco vein mottling virus protease (TVMVp), and sunflower mild mosaic virus protease (SuMMVp) (Fernandez-Rodriguez and Voigt, 2016), were placed under the control of inducible promoters. The expression of these proteases will activate the degradation of target proteins harboring the corresponding tags. By manipulating these components and adjusting the control logic, they built several classic genetic circuits, including inverter and oscillator, and demonstrated the desired dynamic performance. The proteases-mediated dynamic protein degradation was then applied in shikimate and

D-xylonate biosynthesis, with titers reaching 12.63 g/L and 199.44 g/L, respectively (Gao et al., 2019).

Regulation at the protein level is a direct control of protein availability, which is usually faster than regulations at DNA and RNA levels. This type of control is especially efficient for proteins with very long half-lives in cells (Peng et al., 2017). However, control at the protein level does not affect transcription and translation, which means the continued protein synthesis is also a waste of cell resources for unnecessary protein expression, and this may also bring extra burden for the degradation and decrease the regulation efficiencies. Additionally, the limited tunability, relaxed orthogonality, and the lack of protein-protein interaction regulators, may restrain the application of tag-based protein degradation in engineering the biosynthesis of natural product. Therefore, future engineering effort may be taken towards engineering toolsets with expanded tunability and mining new protein degradation mechanisms with improved orthogonality.

2.4 Multilevel regulation

Despite the encouraging advances in using single level regulation for the synthesis of natural compounds, there are some potential pitfalls in each type of regulation as discussed in the previous sections. Therefore, combination of multi-level regulations becomes a growing trend to improve the regulation efficiencies in engineering biosynthesis of natural products, as this can possibly make up for deficiencies in single-level regulation (Calles et al., 2019; Greco et al., 2021; Martínez et al., 2017; Westbrook and Lucks, 2017).

For example, the DNA level regulations can be combined with protein degradation to minimize the regulation time lag by quickly degrading the proteins, and meanwhile, reduce the waste of cell resources for unnecessary protein synthesis by stopping the transcription of target genes. A notable example was seen in the engineering of glucaric acid and myo-inositol production in *E. coli* by combining quorum sensing system and protein degradation to control the transcription and protein availability of selected targets (Gupta et al., 2017). The quorum sensing was used to dynamically down-regulate the transcription of *pfkA* and reduce the glycolytic flux with the cell growth. Meanwhile, a standard ssrA degradation tag was fused to the enzyme Pfk-1 (protein encoded by *pfkA*) to accelerate the depletion of its intracellular availability and quickly shutdown the carbon flux towards glycolysis. This dynamic multilevel regulation enabled a 5.5-fold increase in titers of myo-inositol and pushed the titer of glucaric acid from undetectable to over 0.8 g/L (Gupta et al., 2017). Similar strategy has been applied in the biosynthesis of α-farnesene in *S. cerevisiae* (Yang et al., 2021). In the referred study, the quorum sensing regulators were combined with the auxin-mediated protein degradation system in *S. cerevisiae* to dynamically control the transcription and stability of Erg9, resulting in an 80% increase in the α-farnesene titer (Yang et al., 2021). Multilevel regulation can efficiently make up the deficiencies in single-level regulation, and thus has seen growing applications in engineering biosynthesis of natural products.

While most reported applications of multilevel regulation are combining regulations at DNA- and protein-level, combining DNA level and RNA level regulations, or RNA level and protein level regulations, though were not applied in improving biosynthesis of natural

products, have also been reported. RNA level regulation was combined with protein degradation to modify the protein synthesis rate and stability (Martínez et al., 2017). For multilevel regulations combining DNA and RNA level control, a double feedback circuit with bi-functional control of gene expression was engineered. The engineered circuit involves a translation-inhibitory sRNA with the translational coupling of the target gene to a repressor, and the target gene was under the control of the repressor-regulated promoter (Calles et al., 2019). In another study, Westbrook and co-workers configured and optimized the naturally-derived antisense RNA-mediated transcriptional regulators to control both transcription and translation in *E. coli* (Westbrook and Lucks, 2017). These approaches provide valuable insights for applying the multilevel regulations to achieve tunable gene expression. Thus, we will see more applications of multilevel regulations in the near future with the continuous development and optimizations of these toolsets.

3. Concluding remarks and future perspectives

The biosynthesis of natural products remains a hot topic in metabolic engineering and synthetic biology due to their high value in food, cosmetic, and pharmaceutical industries. As summarized in this review, tuning the gene expression at DNA, RNA, and protein levels via versatile regulation tools has been carried out to effectively improve the synthesis of many natural products. Each level of regulation has its advantages and disadvantages. Thus, multilevel regulation, as a trending regulatory strategy, has seen more and more applications in improving the biosynthesis of natural products, because multilevel regulations can potentially avoid the problems and challenges in using single-level regulation (Calles et al., 2019; Greco et al., 2021; Martínez et al., 2017; Westbrook and Lucks, 2017). Besides the application of improving natural products biosynthesis, the advanced regulatory tools and strategies discussed in this paper can also be employed in engineering production of large protein complexes or non-natural chemicals (Fletcher et al., 2016; Nielsen, 2013), as well as for studying gene regulatory networks (Soma et al., 2017; Wang, T. et al., 2019).

With the expanding availability of biosensors and improvement on gene regulatory toolsets, a rapid increase in applications of dynamic pathway regulation has been observed in engineering biosynthesis of natural products for the last decade. Conventionally, static regulations were carried out to engineer the biosynthesis of natural products due to the ease of manipulation. However, static regulations are one-time, irreversible, and sometimes are manually carried out regardless of the cell status, which would cause undesired performance of microbial cell factories and reduce the productivities (Liu, D. et al., 2018). Dynamic pathway control mediated by genetically encoded biosensors can tune the gene expression and rewire carbon fluxes based on intracellular chemical concentration or environmental signals and thus it can be more efficient when encountering fluctuated chemical concentrations or complex environmental conditions. Thus, it may offer a more flexible and intelligent way to control the biosynthesis of natural products.

While fine-tuning the gene expression sees encouraging progress in engineering natural products, there are some challenges that cannot be fully addressed by these regulatory toolsets and strategies, such as low enzymatic activities or vulnerability to toxic end-products. Therefore, metabolic engineers will see combinatory optimizations that combining

other engineering strategies, such as protein engineering and adaptive laboratory evolution, with the gene expression fine-tuning to improve the biosynthesis of natural products to achieve better titers, yields and productivities in future engineering efforts.

Acknowledgements

This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R35GM128620. We also acknowledge the support from the College of Engineering, The University of Georgia, Athens.

References:

Abernathy MH, He L, Tang YJ, 2017. Channeling in native microbial pathways: Implications and challenges for metabolic engineering. Biotechnology Advances 35(6), 805–814. [PubMed: 28627424]

Aiba H, 2007. Mechanism of RNA silencing by Hfq-binding small RNAs. Curr Opin Microbiol 10(2), 134–139. [PubMed: 17383928]

Ajikumar PK, Xiao W-H, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G, 2010. Isoprenoid pathway optimization for Taxol precursor overproduction in Escherichia coli. Science 330(6000), 70–74. [PubMed: 20929806]

Alper H, Fischer C, Nevoigt E, Stephanopoulos G, 2005. Tuning genetic control through promoter engineering. Proceedings of the National Academy of Sciences of the United States of America 102(36), 12678. [PubMed: 16123130]

Bester AC, Lee JD, Chavez A, Lee Y-R, Nachmani D, Vora S, Victor J, Sauvageau M, Monteleone E, Rinn JL, 2018. An integrated genome-wide CRISPRa approach to functionalize lncRNAs in drug resistance. Cell 173(3), 649–664. e620. [PubMed: 29677511]

Biggs BW, Lim CG, Sagliani K, Shankar S, Stephanopoulos G, De Mey M, Ajikumar PK, 2016. Overcoming heterologous protein interdependency to optimize P450-mediated Taxol precursor synthesis in Escherichia coli. Proc Natl Acad Sci U S A 113(12), 3209–3214. [PubMed: 26951651]

Black WB, Zhang L, Mak WS, Maxel S, Cui Y, King E, Fong B, Sanchez Martinez A, Siegel JB, Li H, 2020. Engineering a nicotinamide mononucleotide redox cofactor system for biocatalysis. Nature Chemical Biology 16(1), 87–94. [PubMed: 31768035]

Calero P, Nikel PI, 2019. Chasing bacterial chassis for metabolic engineering: a perspective review from classical to non-traditional microorganisms. Microbial Biotechnology 12(1), 98–124. [PubMed: 29926529]

Calles B, Goñi-Moreno Á, de Lorenzo V, 2019. Digitalizing heterologous gene expression in Gram-negative bacteria with a portable ON/OFF module. Molecular Systems Biology 15(12), e8777. [PubMed: 31885200]

Cameron DE, Collins JJ, 2014. Tunable protein degradation in bacteria. Nature Biotechnology 32(12), 1276–1281.

Chatterjee P, Jakimo N, Lee J, Amrani N, Rodríguez T, Koseki SRT, Tysinger E, Qing R, Hao S, Sontheimer EJ, Jacobson J, 2020. An engineered ScCas9 with broad PAM range and high specificity and activity. Nature Biotechnology 38(10), 1154–1158.

Chen J, Li W, Yao H, Xu J, 2015. Insights into drug discovery from natural products through structural modification. Fitoterapia 103, 231–241. [PubMed: 25917513]

Chen X, Liu L, 2018. Gene Circuits for Dynamically Regulating Metabolism. Trends Biotechnol 36(8), 751–754. [PubMed: 29352615]

Chouhan S, Sharma K, Zha J, Guleria S, Koffas MAG, 2017. Recent Advances in the Recombinant Biosynthesis of Polyphenols. Frontiers in Microbiology 8, 2259. [PubMed: 29201020]

Christiano R, Nagaraj N, Fröhlich F, Walther Tobias C., 2014. Global Proteome Turnover Analyses of the Yeasts S. cerevisiae and S. pombe. Cell Reports 9(5), 1959–1965. [PubMed: 25466257]

Chung HK, Jacobs CL, Huo Y, Yang J, Krumm SA, Plemper RK, Tsien RY, Lin MZ, 2015. Tunable and reversible drug control of protein production via a self-excising degron. Nature Chemical Biology 11(9), 713–720. [PubMed: 26214256]

Clancy S, 2008. DNA transcription. Nature education 1(1), 41.

Collias D, Beisel CL, 2021. CRISPR technologies and the search for the PAM-free nuclease. Nature Communications 12(1), 555.

Cravens A, Payne J, Smolke CD, 2019. Synthetic biology strategies for microbial biosynthesis of plant natural products. Nat Commun 10(1), 2142. [PubMed: 31086174]

Crocker J, Stern DL, 2013. TALE-mediated modulation of transcriptional enhancers in vivo. Nature methods 10(8), 762. [PubMed: 23817068]

Culver GM, 2001. Meanderings of the mRNA through the Ribosome. Structure 9(9), 751–758. [PubMed: 11566123]

Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, Keasling JD, 2013. Engineering dynamic pathway regulation using stress-response promoters. Nat Biotechnol 31(11), 1039–1046. [PubMed: 24142050]

Deng J, Chen C, Gu Y, Lv X, Liu Y, Li J, Ledesma-Amaro R, Du G, Liu L, 2019. Creating an in vivo bifunctional gene expression circuit through an aptamer-based regulatory mechanism for dynamic metabolic engineering in Bacillus subtilis. Metabolic Engineering 55, 179–190. [PubMed: 31336181]

Dinh CV, Prather KL, 2019. Development of an autonomous and bifunctional quorum-sensing circuit for metabolic flux control in engineered Escherichia coli. Proceedings of the National Academy of Sciences 116(51), 25562–25568.

Doong SJ, Gupta A, Prather KLJ, 2018. Layered dynamic regulation for improving metabolic pathway productivity in Escherichia coli. Proc Natl Acad Sci U S A 115(12), 2964–2969. [PubMed: 29507236]

Ehrenworth AM, Peralta-Yahya P, 2017. Accelerating the semisynthesis of alkaloid-based drugs through metabolic engineering. Nature Chemical Biology 13(3), 249–258. [PubMed: 28199297]

Farmer WR, Liao JC, 2000. Improving lycopene production in Escherichia coli by engineering metabolic control. Nature biotechnology 18(5), 533–537.

Fernandez-Rodriguez J, Voigt CA, 2016. Post-translational control of genetic circuits using Potyvirus proteases. Nucleic acids research 44(13), 6493–6502. [PubMed: 27298256]

Fletcher E, Krivoruchko A, Nielsen J, 2016. Industrial systems biology and its impact on synthetic biology of yeast cell factories. Biotechnology and Bioengineering 113(6), 1164–1170. [PubMed: 26524089]

Fontana J, Sparkman-Yager D, Zalatan JG, Carothers JM, 2020. Challenges and opportunities with CRISPR activation in bacteria for data-driven metabolic engineering. Current Opinion in Biotechnology 64, 190–198. [PubMed: 32599515]

Galanie S, Thodey K, Trenchard IJ, Filsinger Interrante M, Smolke CD, 2015. Complete biosynthesis of opioids in yeast. Science 349(6252), 1095–1100. [PubMed: 26272907]

Gao C, Hou J, Xu P, Guo L, Chen X, Hu G, Ye C, Edwards H, Chen J, Chen W, 2019. Programmable biomolecular switches for rewiring flux in Escherichia coli. Nature communications 10(1), 1–12.

Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC, 2014. Genome-scale CRISPR-mediated control of gene repression and activation. Cell 159(3), 647–661. [PubMed: 25307932]

Greco FV, Pandi A, Erb TJ, Grierson CS, Gorochowski TE, 2021. Harnessing the central dogma for stringent multi-level control of gene expression. Nature Communications 12(1), 1738.

Gupta A, Reizman IM, Reisch CR, Prather KL, 2017. Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nat Biotechnol 35(3), 273–279. [PubMed: 28191902]

Hammer SK, Avalos JL, 2017. Harnessing yeast organelles for metabolic engineering. Nature Chemical Biology 13(8), 823–832. [PubMed: 28853733]

Holmqvist E, Wright PR, Li L, Bischler T, Barquist L, Reinhardt R, Backofen R, Vogel J, 2016. Global RNA recognition patterns of post-transcriptional regulators Hfq and CsrA revealed by UV crosslinking in vivo. EMBO J 35(9), 991–1011. [PubMed: 27044921]

Jang S, Jang S, Xiu Y, Kang TJ, Lee S-H, Koffas MAG, Jung GY, 2017. Development of Artificial Riboswitches for Monitoring of Naringenin In Vivo. ACS Synthetic Biology 6(11), 2077–2085. [PubMed: 28749656]

Jeschek M, Gerngross D, Panke S, 2016. Rationally reduced libraries for combinatorial pathway optimization minimizing experimental effort. Nature Communications 7(1), 11163.

Jiang T, Li C, Teng Y, Zhang R, Yan Y, 2020. Recent advances in improving metabolic robustness of microbial cell factories. Current Opinion in Biotechnology 66, 69–77. [PubMed: 32683192]

Jiang T, Li C, Yan Y, 2021. Optimization of a p-Coumaric Acid Biosensor System for Versatile Dynamic Performance. ACS Synthetic Biology 10(1), 132–144. [PubMed: 33378169]

Jones JA, Toparlak OD, Koffas MA, 2015. Metabolic pathway balancing and its role in the production of biofuels and chemicals. Curr Opin Biotechnol 33, 52–59. [PubMed: 25445548]

Jones JA, Vemacchio VR, Lachance DM, Lebovich M, Fu L, Shirke AN, Schultz VL, Cress B, Linhardt RJ, Koffas MAG, 2015. ePathOptimize: A Combinatorial Approach for Transcriptional Balancing of Metabolic Pathways. Scientific Reports 5(1), 11301. [PubMed: 26062452]

Kaern M, Elston TC, Blake WJ, Collins JJ, 2005. Stochasticity in gene expression: from theories to phenotyoes. Nature Reviews Genetics 6(6), 451–464.

Kang H-S, Charlop-Powers Z, Brady SF, 2016. Multiplexed CRISPR/Cas9- and TAR-Mediated Promoter Engineering of Natural Product Biosynthetic Gene Clusters in Yeast. ACS Synthetic Biology 5(9), 1002–1010. [PubMed: 27197732]

Karin M, 1990. Too many transcription factors: positive and negative interactions. The New Biologist 2(2), 126–131. [PubMed: 2128034]

Keasling JD, 2010. Manufacturing Molecules Through Metabolic Engineering. Science 330(6009), 1355–1358. [PubMed: 21127247]

Kiattisewee C, Dong C, Fontana J, Sugianto W, Peralta-Yahya P, Carothers JM, Zalatan JG, 2021. Portable bacterial CRISPR transcriptional activation enables metabolic engineering in Pseudomonas putida. Metabolic Engineering 66, 283–295. [PubMed: 33930546]

Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517(7536), 583. [PubMed: 25494202]

Kusumawati I, Indrayanto G, 2013. Natural antioxidants in cosmetics, Studies in natural products chemistry. Elsevier, pp. 485–505.

Laity JH, Lee BM, Wright PE, 2001. Zinc finger proteins: new insights into structural and functional diversity. Current Opinion in Structural Biology 11(1), 39–46. [PubMed: 11179890]

Lasa I, Toledo-Arana A, Dobin A, Villanueva M, de los Mozos IR, Vergara-Irigaray M, Segura V, Fagegaltier D, Penadés JR, Valle J, 2011. Genome-wide antisense transcription drives mRNA processing in bacteria. Proceedings of the National Academy of Sciences 108(50), 20172–20177.

Latchman DS, 1993. Transcription factors: an overview. Int J Exp Pathol 74(5), 417–422. [PubMed: 8217775]

Lee ME, Aswani A, Han AS, Tomlin CJ, Dueber JE, 2013. Expression-level optimization of a multi-enzyme pathway in the absence of a high-throughput assay. Nucleic Acids Research 41(22), 10668–10678. [PubMed: 24038353]

Lee S-W, Oh M-K, 2015. A synthetic suicide riboswitch for the high-throughput screening of metabolite production in Saccharomyces cerevisiae. Metabolic Engineering 28, 143–150. [PubMed: 25596509]

Li C, Zhang R, Wang J, Wilson LM, Yan Y, 2020. Protein Engineering for Improving and Diversifying Natural Product Biosynthesis. Trends in Biotechnology 38(7), 729–744. [PubMed: 31954530]

Li S, Prasanna X, Salo VT, Vattulainen I, Ikonen E, 2019. An efficient auxin-inducible degron system with low basal degradation in human cells. Nature methods 16(9), 866–869. [PubMed: 31451765]

Lian J, HamediRad M, Hu S, Zhao H, 2017. Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system. Nat Commun 8(1), 1688. [PubMed: 29167442]

Lian J, Schultz C, Cao M, HamediRad M, Zhao H, 2019. Multi-functional genome-wide CRISPR system for high throughput genotype-phenotype mapping. Nature Communications 10(1), 5794.

Liang C, Zhang X, Wu J, Mu S, Wu Z, Jin J-M, Tang S-Y, 2020. Dynamic control of toxic natural product biosynthesis by an artificial regulatory circuit. Metabolic engineering 57, 239–246. [PubMed: 31837400]

Lim Chin G, Fowler Zachary L, Hueller T, Schaffer S, Koffas Mattheos AG, 2011. High-Yield Resveratrol Production in Engineered Escherichia coli. Applied and Environmental Microbiology 77(10), 3451–3460. [PubMed: 21441338]

Liu D, Mannan AA, Han Y, Oyarzún DA, Zhang F, 2018. Dynamic metabolic control: towards precision engineering of metabolism. Journal of Industrial Microbiology and Biotechnology 45(7), 535–543. [PubMed: 29380150]

Liu Q, Yu T, Li X, Chen Y, Campbell K, Nielsen J, Chen Y, 2019. Rewiring carbon metabolism in yeast for high level production of aromatic chemicals. Nature Communications 10(1), 1–13.

Liu X, Cheng J, Zhang G, Ding W, Duan L, Yang J, Kui L, Cheng X, Ruan J, Fan W, Chen J, Long G, Zhao Y, Cai J, Wang W, Ma Y, Dong Y, Yang S, Jiang H, 2018. Engineering yeast for the production of breviscapine by genomic analysis and synthetic biology approaches. Nature Communications 9(1).

Liu Y, Nielsen J, 2019. Recent trends in metabolic engineering of microbial chemical factories. Current Opinion in Biotechnology 60, 188–197. [PubMed: 31185380]

Liu Y, Wan X, Wang B, 2019. Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria. Nature Communications 10(1), 3693.

Lu Z, Peng B, Ebert BE, Dumsday G, Vickers CE, 2021. Auxin-mediated protein depletion for metabolic engineering in terpene-producing yeast. Nature Communications 12(1), 1051.

Mahr R, Frunzke J, 2016. Transcription factor-based biosensors in biotechnology: current state and future prospects.

Mandal M, Boese B, Barrick JE, Winkler WC, Breaker RR, 2003. Riboswitches Control Fundamental Biochemical Pathways in Bacillus subtilis and Other Bacteria. Cell 113(5), 577–586. [PubMed: 12787499]

Martínez V, Lauritsen I, Hobel T, Li S, Nielsen AT, Nørholm Morten H.H., 2017. CRISPR/Cas9-based genome editing for simultaneous interference with gene expression and protein stability. Nucleic Acids Research 45(20), e171–e171. [PubMed: 28981713]

Miyahisa I, Funa N, Ohnishi Y, Martens S, Moriguchi T, Horinouchi S, 2006. Combinatorial biosynthesis of flavones and flavonols in Escherichia coli. Appl Microbiol Biotechnol 71(1), 53–58. [PubMed: 16133333]

Mizuno T, Chou MY, Inouye M, 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proceedings of the National Academy of Sciences 81(7), 1966–1970.

Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY, 2013. Metabolic engineering of Escherichia coli using synthetic small regulatory RNAs. Nat Biotechnol 31(2), 170–174. [PubMed: 23334451]

Naseri G, Behrend J, Rieper L, Mueller-Roeber B, 2019. COMPASS for rapid combinatorial optimization of biochemical pathways based on artificial transcription factors. Nature Communications 10(1), 2615.

Ni J, Zhang G, Qin L, Li J, Li C, 2019. Simultaneously down-regulation of multiplex branch pathways using CRISPRi and fermentation optimization for enhancing β-amyrin production in Saccharomyces cerevisiae. Synthetic and Systems Biotechnology 4(2), 79–85. [PubMed: 30949594]

Nielsen J, 2013. Production of biopharmaceutical proteins by yeast. Bioengineered 4(4), 207–211. [PubMed: 23147168]

Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M, 2009. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. Nature methods 6(12), 917–922. [PubMed: 19915560]

Palmer CM, Miller KK, Nguyen A, Alper HS, 2019. Engineering 4-coumaroyl-CoA derived polyketide production in Yarrowia lipolytica through a β-oxidation mediated strategy. Metabolic Engineering.

Park SY, Yang D, Ha SH, Lee SY, 2018. Metabolic Engineering of Microorganisms for the Production of Natural Compounds. Advanced Biosystems 2(1).

Pelechano V, Steinmetz LM, 2013. Gene regulation by antisense transcription. Nature Reviews Genetics 14(12), 880–893.

Peng B, Nielsen LK, Kampranis SC, Vickers CE, 2018. Engineered protein degradation of farnesyl pyrophosphate synthase is an effective regulatory mechanism to increase monoterpene production in Saccharomyces cerevisiae. Metabolic Engineering 47(6 2017), 83–93.

Peng B, Plan MR, Chrysanthopoulos P, Hodson MP, Nielsen LK, Vickers CE, 2017. A squalene synthase protein degradation method for improved sesquiterpene production in Saccharomyces cerevisiae. Metab Eng 39, 209–219. [PubMed: 27939849]

Peng R, Wang Y, Feng W.-w., Yue X.-j., Chen J.-h., Hu X.-z., Li Z.-f, Sheng D.-h., Zhang Y.-m., Li Y.-z., 2018. CRISPR/dCas9-mediated transcriptional improvement of the biosynthetic gene cluster for the epothilone production in Myxococcus xanthus. Microbial Cell Factories 17(1), 15. [PubMed: 29378572]

Pfeiffer F, Mayer G, 2016. Selection and biosensor application of aptamers for small molecules. Frontiers in chemistry 4, 25. [PubMed: 27379229]

Rodrigues T, Reker D, Schneider P, Schneider G, 2016. Counting on natural products for drug design. Nat Chem 8(6), 531–541. [PubMed: 27219696]

Rugbjerg P, Myling-Petersen N, Porse A, Sarup-Lytzen K, Sommer MOA, 2018. Diverse genetic error modes constrain large-scale bio-based production. Nature Communications 9(1), 787–787.

Ruscito A, DeRosa MC, 2016. Small-molecule binding aptamers: Selection strategies, characterization, and applications. Frontiers in chemistry 4, 14. [PubMed: 27242994]

Salehi B, Fokou PV, Sharifi-Rad M, Zucca P, Pezzani R, Martins N, Sharifi-Rad J, 2019. The Therapeutic Potential of Naringenin: A Review of Clinical Trials. Pharmaceuticals 12(1).

Sandberg TE, Salazar MJ, Weng LL, Palsson BO, Feist AM, 2019. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. Metabolic Engineering 56, 1–16. [PubMed: 31401242]

Sander T, Wang CY, Glatter T, Link H, 2019. CRISPRi-based downregulation of transcriptional feedback improves growth and metabolism of arginine overproducing E. coli. ACS synthetic biology 8(9), 1983–1990. [PubMed: 31429546]

Sekar K, Gentile AM, Bostick JW, Tyo KE, 2016. N-terminal-based targeted, inducible protein degradation in Escherichia coli. PLoS One 11(2), e0149746. [PubMed: 26900850]

Shen X, Wang J, Li C, Yuan Q, Yan Y, 2019. Dynamic gene expression engineering as a tool in pathway engineering. Current opinion in biotechnology 59, 122–129. [PubMed: 31063878]

Soma Y, Fujiwara Y, Nakagawa T, Tsuruno K, Hanai T, 2017. Reconstruction of a metabolic regulatory network in Escherichia coli for purposeful switching from cell growth mode to production mode in direct GABA fermentation from glucose. Metabolic Engineering 43(July), 54–63. [PubMed: 28800966]

Svensson SL, Sharma CM, 2016. Small RNAs in bacterial virulence and communication. Virulence Mechanisms of Bacterial Pathogens, 169–212.

Tsui H-CT, Leung H-CE, Winkler ME, 1994. Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in Escherichia coli K-12. Molecular Microbiology 13(1), 35–49. [PubMed: 7984093]

Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N, 2007. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. Biotechnol J 2(10), 1214–1234. [PubMed: 17935117]

Vogel J, Wagner EGH, 2007. Target identification of small noncoding RNAs in bacteria. Current Opinion in Microbiology 10(3), 262–270. [PubMed: 17574901]

Wang J, Cui X, Yang L, Zhang Z, Lv L, Wang H, Zhao Z, Guan N, Dong L, Chen R, 2017. A real-time control system of gene expression using ligand-bound nucleic acid aptamer for metabolic engineering. Metabolic Engineering 42, 85–97. [PubMed: 28603040]

Wang J, Gao D, Yu X, Li W, Qi Q, 2015. Evolution of a chimeric aspartate kinase for L-lysine production using a synthetic RNA device. Applied microbiology and biotechnology 99(20), 8527–8536. [PubMed: 25935345]

Wang J, Shen X, Rey J, Yuan Q, Yan Y, 2018. Recent advances in microbial production of aromatic natural products and their derivatives. Appl Microbiol Biotechnol 102(1), 47–61. [PubMed: 29127467]

Wang R, Cress BF, Yang Z, Hordines JC, Zhao S, Jung GY, Wang Z, Koffas MAG, 2019. Design and Characterization of Biosensors for the Screening of Modular Assembled Naringenin Biosynthetic Library in Saccharomyces cerevisiae. ACS Synthetic Biology 8(9), 2121–2130. [PubMed: 31433622]

Wang R, Zhao S, Wang Z, Koffas MAG, 2020. Recent advances in modular co-culture engineering for synthesis of natural products. Current Opinion in Biotechnology 62, 65–71. [PubMed: 31605875]

Wang T, Zheng X, Ji H, Wang T-L, Xing X-H, Zhang C, 2019. Dynamics of transcription-translation coordination tune bacterial indole signaling. Nature Chemical Biology 2019, 1–10.

Wang W, Li S, Li Z, Zhang J, Fan K, Tan G, Ai G, Lam SM, Shui G, Yang Z, Lu H, Jin P, Li Y, Chen X, Xia X, Liu X, Dannelly HK, Yang C, Yang Y, Zhang S, Alterovitz G, Xiang W, Zhang L, 2020. Harnessing the intracellular triacylglycerols for titer improvement of polyketides in Streptomyces. Nature Biotechnology 38(1), 76–83.

Wang Z, Cirino PC, 2016. New and improved tools and methods for enhanced biosynthesis of natural products in microorganisms. Current Opinion in Biotechnology 42, 159–168. [PubMed: 27284635]

Weissman KJ, 2016. Genetic engineering of modular PKSs: from combinatorial biosynthesis to synthetic biology. Nat. Prod. Rep 33(2), 203–230. [PubMed: 26555805]

Westbrook AM, Lucks JB, 2017. Achieving large dynamic range control of gene expression with a compact RNA transcription-translation regulator. Nucleic Acids Research 45(9), 5614–5624. [PubMed: 28387839]

Wu G, Yan Q, Jones JA, Tang YJ, Fong SS, Koffas MA, 2016. Metabolic burden: cornerstones in synthetic biology and metabolic engineering applications. Trends in biotechnology 34(8), 652–664. [PubMed: 26996613]

Wu J, Du G, Chen J, Zhou J, 2015. Enhancing flavonoid production by systematically tuning the central metabolic pathways based on a CRISPR interference system in Escherichia coli. Scientific reports 5(1), 1–14.

Wu J, Yu O, Du G, Zhou J, Chen J, 2014. Fine-Tuning of the Fatty Acid Pathway by Synthetic Antisense RNA for Enhanced (2S)-Naringenin Production from l-Tyrosine in Escherichia coli. Appl Environ Microbiol 80(23), 7283–7292. [PubMed: 25239896]

Wu Y, Chen T, Liu Y, Tian R, Lv X, Li J, Du G, Chen J, Ledesma-Amaro R, Liu L, 2020. Design of a programmable biosensor-CRISPRi genetic circuits for dynamic and autonomous dual-control of metabolic flux in Bacillus subtilis. Nucleic Acids Research 48(2), 996–1009. [PubMed: 31799627]

Xiu Y, Jang S, Jones JA, Zill NA, Linhardt RJ, Yuan Q, Jung GY, Koffas MAG, 2017. Naringenin-responsive riboswitch-based fluorescent biosensor module for Escherichia coli co-cultures. Biotechnology and Bioengineering 114(10), 2235–2244. [PubMed: 28543037]

Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M, 2014. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc Natl Acad Sci U S A 111(31), 11299–11304. [PubMed: 25049420]

Yang D, Park SY, Park YS, Eun H, Lee SY, 2020. Metabolic Engineering of Escherichia coli for Natural Product Biosynthesis. Trends in Biotechnology 38(7), 745–765. [PubMed: 31924345]

Yang D, Yoo SM, Gu C, Ryu JY, Lee JE, Lee SY, 2019. Expanded synthetic small regulatory RNA expression platforms for rapid and multiplex gene expression knockdown. Metabolic Engineering 54, 180–190. [PubMed: 30999052]

Yang J, Seo SW, Jang S, Shin S-I, Lim CH, Roh T-Y, Jung GY, 2013. Synthetic RNA devices to expedite the evolution of metabolite-producing microbes. Nature Communications 4(1), 1413.

Yang X, Liu J, Zhang J, Shen Y, Qi Q, Bao X, Hou J, 2021. Quorum sensing-mediated protein degradation for dynamic metabolic pathway control in Saccharomyces cerevisiae. Metabolic Engineering 64, 85–94. [PubMed: 33545357]

Yang Y, Lin Y, Li L, Linhardt RJ, Yan Y, 2015. Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products. Metab Eng 29, 217–226. [PubMed: 25863265]

Yang Y, Lin Y, Wang J, Wu Y, Zhang R, Cheng M, Shen X, Wang J, Chen Z, Li C, Yuan Q, Yan Y, 2018. Sensor-regulator and RNAi based bifunctional dynamic control network for engineered microbial synthesis. Nat Commun 9(1), 3043. [PubMed: 30072730]

Yoo SM, Na D, Lee SY, 2013. Design and use of synthetic regulatory small RNAs to control gene expression in Escherichia coli. Nature Protocols 8(9), 1694–1707. [PubMed: 23928502]

Yuan J, Ching C-B, 2015. Dynamic control of ERG9 expression for improved amorpha-4,11-diene production in Saccharomyces cerevisiae. Microbial Cell Factories 14(1), 38. [PubMed: 25889168]

Zha J, Wu X, Koffas MAG, 2020. Making brilliant colors by microorganisms. Current Opinion in Biotechnology 61, 135–141. [PubMed: 31927454]

Zhang R, Li C, Wang J, Yang Y, Yan Y, 2018. Microbial production of small medicinal molecules and biologics: From nature to synthetic pathways. Biotechnol Adv 36(8), 2219–2231. [PubMed: 30385278]

Zhao P, Li Q, Tian P, Tan T, 2021. Switching metabolic flux by engineering tryptophan operon-assisted CRISPR interference system in Klebsiella pneumoniae. Metabolic Engineering.

Zhao S, Jones JA, Lachance DM, Bhan N, Khalidi O, Venkataraman S, Wang Z, Koffas MAG, 2015. Improvement of catechin production in Escherichia coli through combinatorial metabolic engineering. Metabolic Engineering 28, 43–53. [PubMed: 25527438]

Zhao X, Shi F, Zhan W, 2015. Overexpression of ZWF1 and POS5 improves carotenoid biosynthesis in recombinant Saccharomyces cerevisiae. Letters in Applied Microbiology 61(4), 354–360. [PubMed: 26179622]

Zhou L-B, Zeng A-P, 2015. Engineering a Lysine-ON Riboswitch for Metabolic Control of Lysine Production in Corynebacterium glutamicum. ACS Synthetic Biology 4(12), 1335–1340. [PubMed: 26300047]

Zhou S, Lyu Y, Li H, Koffas MAG, Zhou J, 2019. Fine-tuning the (2S)-naringenin synthetic pathway using an iterative high-throughput balancing strategy. Biotechnology and Bioengineering 116(6), 1392–1404. [PubMed: 30684358]

Zhu L.-y., Qiu X.-y., Zhu L.-y., Wu X.-m., Zhang Y, Zhu Q.-h., Fan D.-y., Zhu C.-s., Zhang D.-y., 2016. Spatial organization of heterologous metabolic system in vivo based on TALE. Scientific Reports 6(1), 26065. [PubMed: 27184291]

Web reference:

Global Vanillin Market Size, Grandview Research. https://www.grandviewresearch.com/industry-analysis/vanillin-market (accessed 27th April)

Highlights:

- Constructing efficient microbial cell factories for synthesizing natural products requires fine-tuning the gene expression.
- Three typical regulation levels (DNA, RNA, and protein) were included, and the commonly used toolsets in these levels were discussed in detail.
- Combining multi-level regulations, transition from static control to dynamic regulation, and expanding current regulation toolset repertoire, present future opportunities to improve the efficiencies of gene regulation.

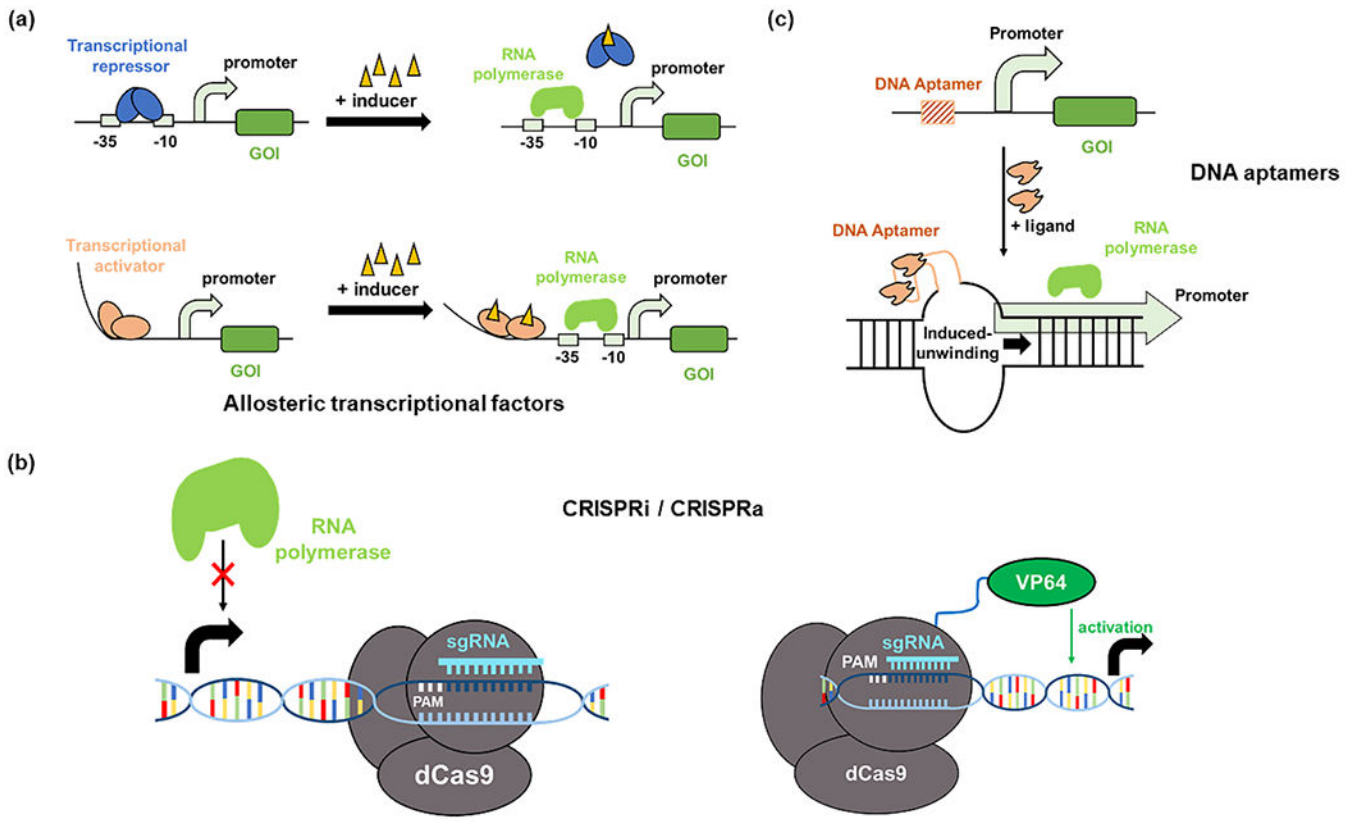

Figure 1.

Schemes of DNA-level regulation toolsets. **(a)** Regulations mediated by transcriptional repressors (up) and transcriptional activators (down). Transcriptional repressors will occupy the −35 and −10 regions, thus blocking the access of RNA polymerase. When the inducer is present, the repressor can bind to the inducer. This process will result in a conformational change of the repressor, making it unable to bind the DNA sequence and thus release the transcriptional inhibition. For the transcriptional activator, the regulatory protein can bind to the DNA and force the DNA to bend. When the inducer is present, the binding of the inducer can cause a conformational change of the activator, which consequently leads to interaction with the RNA polymerase, and this will activate the expression of the target gene. GOI, gene of interest. **(b)** CRISPR based gene interference (CRISPRi, left) and activation (CRISPRa, right). For CRISPRi, the sgRNA (single guide RNA) will guide the dCas9 to the promoter region, blocking the access of RNA polymerase to the promoter, and thus inhibiting the transcription. For CRISPRa, an activation domain (e.g., VP64, derived from the herpes simplex virus) is fused to dCas9 protein, and the sgRNA can guide the dCas9 to the appropriate location and bring the activator into contact with RNA polymerase. Upon the interaction, the promoter can be activated. PAM, protospacer adjacent motif, is a short DNA sequence that follows the DNA region targeted for cleavage by the CRISPR system. **(c)** Regulations mediated by DNA aptamers. The binding of ligand would cause unwinding of double stranded DNA. This would allow easier recognition and binding by RNA polymerases if the aptamer is placed near the promoter region, leading to the enhanced expression of downstream genes at the transcriptional level.

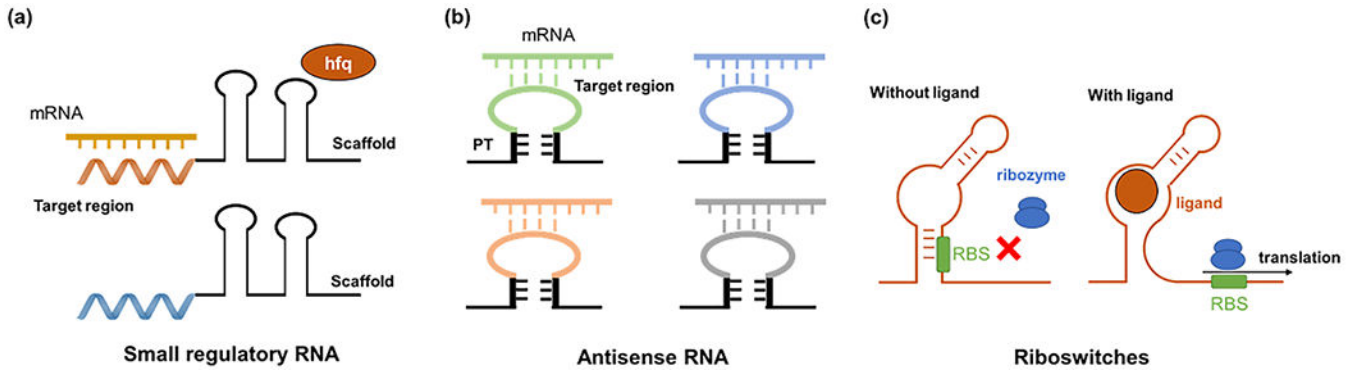

Figure 2.

Schemes of RNA-level regulation toolsets. **(a)** Regulations mediated by small regulatory RNAs. A scaffold is used to enhance the stability of sRNA. A short binding sequence (target region) that can bind with target mRNA by complementary pairing and hinder the translation. Two small regulatory RNAs indicate that the target region is programmable/designable based on different regulation sequence but the scaffold can be similar. The scaffolds presented here for the two sRNAs (in black) are identical. Hfq, an abundant bacterial RNA binding protein that is physiologically important in interacting with sRNA. **(b)** Regulations mediated by antisense RNAs. Similar to sRNA, the asRNA achieves the regulation via complementary pairing with target mRNA. A 100 bp pair termini (PT) structure (in black) is used to stabilize the asRNA. The different colors in the asRNA represent varied target regions that can bind with different sequences. **(c)** Regulations mediated by riboswitches. The RNA secondary structure of riboswitch can block the access of ribozyme to the ribozyme binding site (RBS) and thus hinder the translation. This inhibition will be released upon ligand binding, and the ribozyme can then recognize the exposed RBS and start the translation.

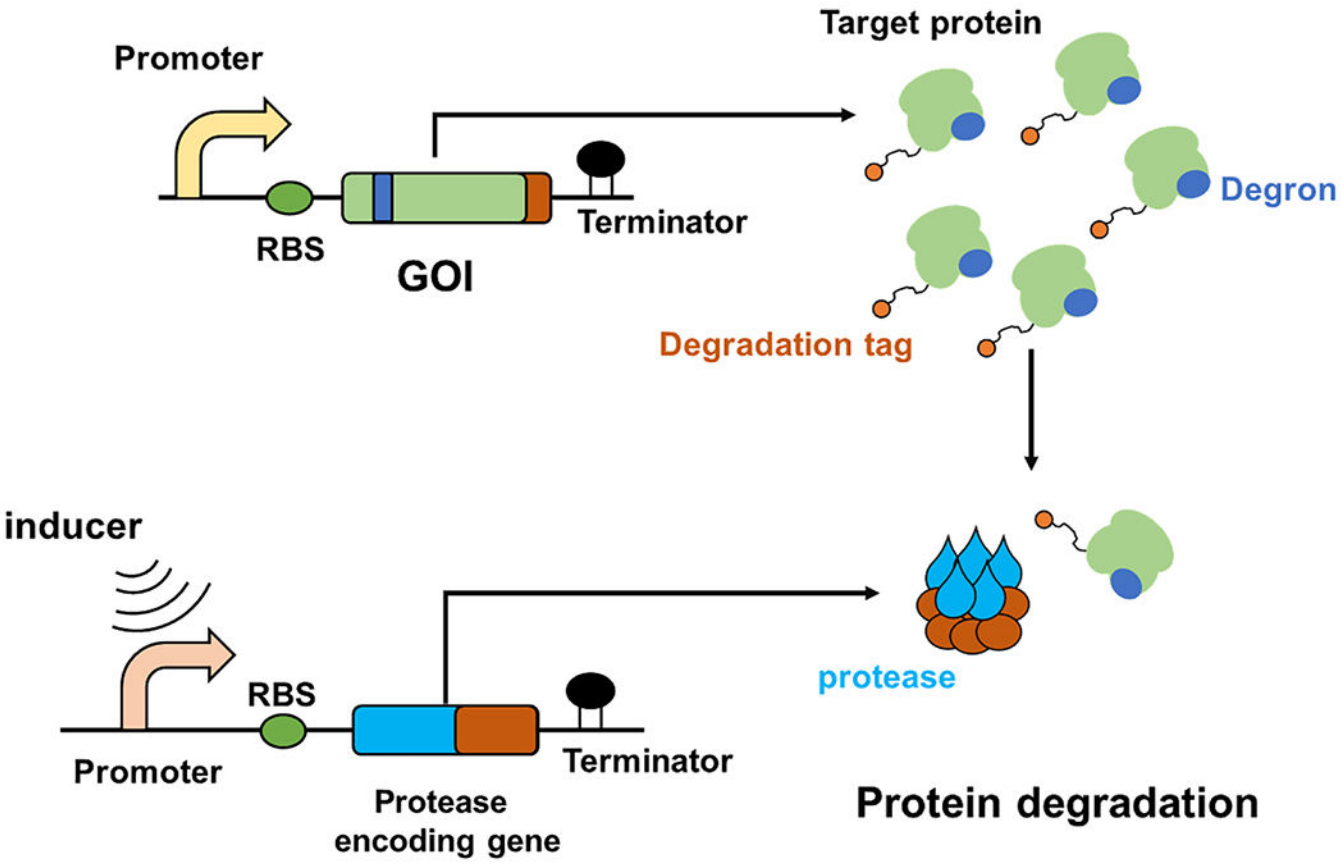

Figure 3.

Schemes of protein-level regulation. Regulations at protein level are usually achieved by introducing the degradation tags into target proteins. The blue rectangle in the GOI represents the DNA sequence of degron, and the brown rectangle near the GOI represents the coding sequence of the degradation tag. The tagged protein will be recognized by corresponding protease and then degraded. The protease usually contains two functional domains, one for tag recognition (in blue) and one for degrading the tagged protein (in brown). For dynamic protein degradation, the protease can also be controlled by an inducible promoter (in pink) to enable inducible protein degradation. GOI, gene of interest.

Table 1

Summary of notable examples in fine-tuning gene expression to improve the biosynthesis of natural products.

Compounds (hosts [a])	Tool/strategy used (regulation level)	Achievements [b]	Reference [c]
Naringenin (*S.c*)	promoter engineering (DNA)	Increased the final titer to 52.0 mg/L	(Lee et al., 2013)
Naringenin (*E.c*)	promoter engineering (DNA)	A 2.1-fold increase in the final titer (191 mg/L)	(Zhou et al., 2019)
Lycopene (*E.c*)	aTFs (DNA)	Increased the final titer to over 150 mg/L	(Farmer and Liao, 2000)
Amorphadiene (*E.c*)	aTFs (DNA)	A 2-fold increase in the final titer (1.6 g/L)	(Dahl et al., 2013)
Fatty acids (*E.c*)	aTFs (DNA)	A 15.7-fold increase in the final titer (3.86 g/L) compared to wild-type strain	(Xu et al., 2014)
β-Ionone (*S.c*)	aTFs (DNA)	A 4.2-fold increase in the final titer compared to wild-type strain	(Naseri et al., 2019)
Salicylic acid (*E.c*)	aTFs (DNA)	A 1.8-fold improvement in the final titer (520 mg/L)	(Dinh and Prather, 2019)
Vanillin (*E.c*)	aTFs (DNA)	Increased the final titer to 1.5 g/L	(Liang et al., 2020)
Naringenin (*E.c*)	CRISPRi (DNA)	A 7.6-fold increase in the final titer (421.6 mg/L)	(Wu et al., 2015)
β-Amyrin (*S.c*)	CRISPRi (DNA)	A 44.3% increase in the final titer (156.7 mg/L)	(Ni et al., 2019)
Arginine (*E.c*)	CRISPRi (DNA)	A 2-fold increase in growth rate with similar arginine production	(Sander et al., 2019)
Epothilone (*M.x*)	CRISPRa (DNA)	Increased the final titer of to over 25 mg/L	(Peng, R. et al., 2018)
Biopterin (*P.p*)	CRISPRa (DNA)	A more than 5-fold increase in the final titer	(Kiattisewee et al., 2021)
Mevalonate (*P.p*)	CRISPRa (DNA)	A 40-fold increases in the final titer (402 mg/L)	
β-Carotene (*S.c*)	Multiplex-CRISPR (DNA)	A 2.8-fold increase in the final titer	(Lian et al., 2017)
2′-Fucosyllactose (*B.s*)	DNA aptamer (DNA)	A 22.3-fold increase in the final titer (511 mg/L)	(Deng et al., 2019)
Inole-3-acetic acid (*E.c*)	TALE (DNA)	A 9.6-fold increase in the final titer	(Zhu et al., 2016)
Cadaverine (*E.c*)	Small regulatory RNA (RNA)	A 55% increase in the final titer	(Na et al., 2013)
L-Threonine (*E.c*)	Small regulatory RNA (RNA)	Increased the final titer to 22.9 g/L	(Yang et al., 2019)
L-Proline (*E.c*)	Small regulatory RNA (RNA)	Increased the final titer to 54.1 g/L	
Crude violacein (*E.c*)	Small regulatory RNA (RNA)	Increased the final titer to 5.19 g/L	
Indigo (*E.c*)	Small regulatory RNA (RNA)	Increased the final titer to 135 mg/L	
Naringenin (*E.c*)	Antisense RNA (RNA)	A 4.31-fold increase in the final titer (391 mg/L)	(Wu et al., 2014)
Naringenin (*E.c*)	Antisense RNA (RNA)	A 1.53-fold increase in the final titer (91.31 mg/L)	(Yang et al., 2015)
4-Hydroxycoumarin (*E.c*)	Antisense RNA (RNA)	A 2.53-fold increase in the final titer (270.85 mg/L)	
Resveratrol (*E.c*)	Antisense RNA (RNA)	A 1.70-fold increase in the final titer (268.20 mg/L)	
L-Lysine (*E.c*)	Riboswitch (RNA)	An 89% increase in the final yield	(Zhou and Zeng, 2015)
2′-Fucosyllactose (*B.s*)	Riboswitch (RNA)	A 27.3-fold increase in the final titer (674 mg/L)	(Deng et al., 2019)
Nerolidol (*S.c*)	PEST Degron (Protein)	An 86% increase in the final titer	(Peng et al., 2017)
Linalool (*S.c*)	Degron (Protein)	A 27-fold increase in the final titer (11 mg/L)	(Peng, B. et al., 2018)

Compounds (hosts [a])	Tool/strategy used (regulation level)	Achievements [b]	Reference [c]
Shikimate (*E.c*)	Dynamic heterologous proteases expression (Protein)	Increased the titer to 12.63 g/L	(Gao et al., 2019)
D-Xylonate (*E.c*)	Dynamic heterologous proteases expression (Protein)	Increased the titer to 199.44 g/L	
Limonene (*S.c*)	Auxin-inducible degradation (Protein)	Increased the final titer to 76 mg/L	(Lu et al., 2021)
Nerolidol (*S.c*)	Auxin-inducible degradation (Protein)	Increased the final titer to 3.5 g/L	
Glucaric acid (*E.c*)	Multilevel regulations: quorum sensing (DNA) with protein degradation (Protein)	Increased the final titer from undetectable to over 0.8 g/L	(Gupta et al., 2017)
Myo-Inositol (*E.c*)	Multilevel regulations: quorum sensing (DNA) with protein degradation (Protein)	A 5.5-fold increase in the final titer	
α-Farnesene (*S.c*)	Multilevel regulations: quorum sensing (DNA) with protein degradation (Protein)	An 80% increase in the final titer	(Yang et al., 2021)

[a]The abbreviations for hosts listed in the table: E.c, *Escherichia coli*; S.c, *Saccharomyces cerevisiae*; B.s, *Bacillus subtilis*; P.p, *Pseudomonas putida*; M.x, *Myxococcus xanthus*.

[b]All fold-change were calculated based on the titer of the unregulated control unless specified in the table. The unregulated control means the strain with the same genotype and pathway but was not regulated by the toolset/strategy.

[c]When there were multiple examples demonstrated in one reference, the reference cells were merged to indicate that these examples/chemicals were using the same reference.