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Transcription factor-based biosensors: A molecular-guided approach for natural product engineering

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Abstract

Natural products and their derivatives offer a rich source of chemical and biological diversity; however, traditional engineering of their biosynthetic pathways to improve yields and access to unnatural derivatives requires a precise understanding of their enzymatic processes. High-throughput screening platforms based on allosteric transcription-factor based biosensors can be leveraged to overcome the screening bottleneck to enable searching through large libraries of pathway/strain variants. Herein, the development and application of engineered allosteric transcription factor-based biosensors is described that enable optimization of precursor availability, product titers, and downstream product tailoring for advancing the natural product bioeconomy. We discuss recent successes for tailoring biosensor design, including computationally-based approaches, and present our future outlook with the integration of cell-free technologies and *de novo* protein design for rapidly generating biosensor tools.

Graphical Abstract

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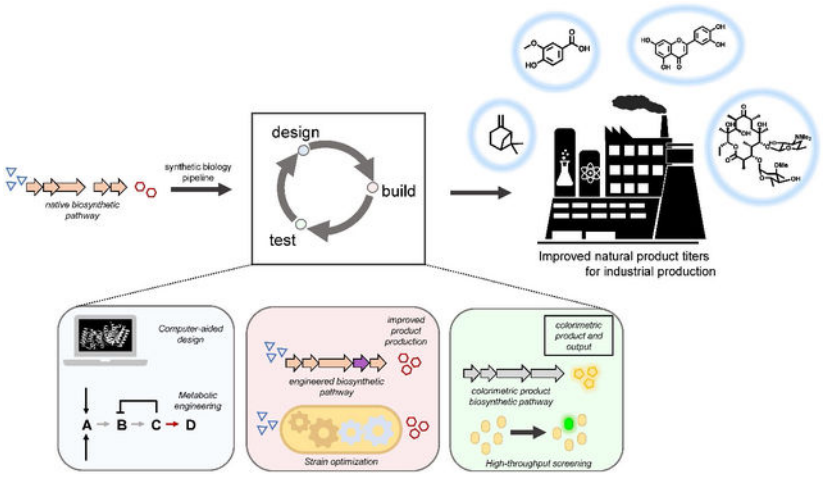
Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Introduction

Natural products represent privileged scaffolds for the development of pharmaceuticals, as well as numerous high-value commodity and industrial chemicals in the growing bioeconomy. Industrial strategies to produce these secondary metabolites (e.g. alkaloids, phenylpropanoids, polyketides, and terpenoids) combine various approaches including chemical synthesis, biocatalysis, and extraction from natural sources [1]. As industries shift towards increasingly environmentally sustainable and cost-effective methods, it is no surprise that the enzyme industry reported a total added value of $106 billion to the US economy in 2016 [2].

Microbial metabolism has been harnessed to provide access to natural products and their analogues via chemo- and regioselective modification of complex organic scaffolds that is otherwise often challenging to achieve through traditional synthetic methods. However, engineering of enzymes, metabolic pathways, and microbial hosts are often required to optimize substrate scope, cofactor recycling, catalytic rate, substrate tolerance, and productivity within stringent industrial conditions [1]. The framework of synthetic biology's iterative "design-build-test" cycle has enabled rapid engineering of microbial metabolism, leading to significant improvements in the design and construction of libraries. However, screening these libraries for variants with desired properties remains a critical bottleneck and highlights the need for high-throughput approaches that provide a rapid link between genotype and phenotype [3,4]. Genetically-encoded biosensors based on allosteric transcription factors (aTFs) that detect the structure and report the titer of a given natural product allow for the programmable high-throughput screening and selection of genetic constructs, host strains, and experimental growth conditions (Fig. 1).

Ubiquitous in nature, aTFs activate or repress transcriptional machinery to regulate gene expression in response to environmental stimuli. The ligand-binding domain (LBD) of metabolite-responsive aTFs selectively bind effector molecules, which results in an allosterically induced conformational change that modulates transcription through the interactions of the aTF DNA-binding domain (DBD) and its cognate DNA operator

sequence (Fig. 2a). Characterization of these proteins has resulted in the development of diverse aTF-based tools. The properties of these regulatory factors, including their inducer specificity as well as their various transfer functions as defined by the Hill equation, including sensitivity ($K_{1/2}$), dynamic range (ON-OFF), and cooperativity (n), can be tailored to yield an optimized biosensor. aTFs respond to effector structures and are therefore completely agnostic to biological activity or mechanism of action of the natural product. Coupled with the ability to engineer aTFs with exquisite effector selectivity, aTFs present a unique opportunity to develop biosensors for a variety of natural products with molecular precision sufficient to guide the regulation and engineering of complex biosynthetic systems. Recent reports have leveraged these platforms for dynamic pathway regulation to balance the flux of intermediates and final products. In tandem with strain development, modularized gene expression, dynamic regulation, and other metabolic engineering strategies, engineered enzymes can be exploited to produce high-value natural products and their derivatives [5]. Herein, this review highlights the advancements in biosensor-guided approaches to natural product engineering over the last two years, with a special emphasis on aTF platforms for optimizing precursors, pathways, final titers, and post-assembly tailoring. Finally, we reflect on the future outlook of these tools.

Development and Engineering of aTF-Based Biosensors

While approaches to mine and develop endogenous aTFs into biosensors have been successful, there are a limited number of characterized aTFs known in nature with substrate scopes that are relevant to natural products (Table 1). As such, recent studies have employed genomic sequencing to expand the biosensor platform repertoire by identifying previously uncharacterized aTFs for specific final products, including the phenylpropanoid resveratrol [6], and the steroid progesterone [7]. However, when a suitable native aTF cannot be identified, the ligand specificity of an aTF can be expanded via directed evolution to create designer biosensor platforms (Fig. 2b) by coupling rounds of mutagenesis [8-11] with high-throughput techniques such as fluorescence activated cell sorting (FACS) or antibiotic selection [6,12-14]. For example, Kasey *et al* engineered MphR, a promiscuous macrolide sensing aTF, to expand its promiscuity towards various natural and non-natural macrolides that were otherwise poor effectors of the wild-type MphR [12]. Similarly, a chimeric LysR biosensor was developed to selectively detect luteolin from three closely related flavonoids, naringenin, apigenin, and luteolin, by exploring a variety of chimeric detector-effector pairs [10]. The novel chimeric biosensor displayed stringent specificity for luteolin and is the first reported luteolin-specific transcriptional biosensor in *Escherichia coli* (*E. coli*) [10]. The protocatechuic acid (PCA) biosensor PcaV was evolved to alter its ligand specificity towards vanillin and other close aromatic aldehydes, generating the Van2 biosensor [11]. Mutational analysis revealed that the combination of mutations M113S/N114A was sufficient for the vanillin specificity, while I110V played an important role for the reduction of basal expression and stabilization [11]. The dynamic range of the Van2 biosensor with vanillin was 7.7-fold, while being unresponsive to the parental aromatic acid, PCA [11].

Tailoring the sensitivity and dynamic range of aTFs often relies on regulating the intracellular concentration of the aTF through the engineering of *cis*-regulatory components, such as the promoter or ribosome binding site (RBS), which control the rate of transcription

and translation, respectively. Engineering of these components can be accomplished by semi-rational design with tools like '*De Novo* DNA', an open-access online RBS and operon calculator that predicts translation initiation rates [35]. Alternatively, this can be achieved by random mutagenesis and screening. For instance, the dynamic range of MphR, a macrolide-sensing aTF, was improved 10-fold with randomized mutagenesis of the protein's RBS sequence [12]. Dynamic range can also be fine-tuned by engineering the promoter sequence to reduce or eliminate undesired background noise, leaky expression, and varied limit of detection [36]. Beyond engineering of the repressor module, the sensitivity of an aTF biosensor can also be modulated by altering the location of the operator sequence within the promoter region of the reporter module [34]. However, mechanistic understanding of the interactions between aTFs and their corresponding regulatory components are typically not fully characterized or standardized. By leveraging a combination of RBS and promoter engineering, transcription and translation are controlled at the most fundamental level, enabling better control and success of aTF-biosensing platforms.

Computational Approaches to Biosensor Design

The success of aTF engineering via random or semi-random mutagenesis notwithstanding, computational platforms have leveraged statistical and mathematical modeling to create designer aTF-based biosensors and have tuned their transfer functions within complex gene regulatory networks. Recently, 'design of experiments', a statistical modeling system using structured, multivariate experimentation, was leveraged to map gene expression levels and tailor the Hill parameters of a protocatechuic acid biosensor [37]. Similarly, mathematical models have been applied to customize biosensor components to alter aTF biosensor activation thresholds, sensitivities, selectivity's and dynamic ranges [38-40]. Quantitative modeling strategies, such as those developed by Swank *et al*, have been applied to characterize a library of synthetic transcription factors and their corresponding promoters, which were subsequently used to engineer and build *de novo* transcriptional regulatory networks [41].

Recent advances in biosensor engineering to create designer biosensors have also been accessed via structural modeling *in silico* [30]. Computational LBDs for *de novo* aTFs are expected to spur the next generation of biosensor development by providing exquisite affinity and selectivity for specific target molecules that model the same characteristics as naturally occurring aTFs [42]. As a result of improved computing power and the low cost of DNA synthesis, computationally designed *de novo* proteins are expected to revolutionize the next generation of biosensor development as they explore the full sequence space of possible amino acids [43]. For example, a small molecule biosensor was constructed from the computationally designed digoxigenin LBD [44]. Conditionally destabilized mutations in the digoxigenin LBD created a functional biosensor that is unstable unless bound to its new target effector, progesterone, thereby transforming a *de novo* LBD into a biosensor platform with unavailable or unknown aTFs [44]. It is anticipated that computational protein design and mathematical modeling will improve access to a broader range of molecular sensors, including those for natural products.

Biosensor-Guided Optimization of Precursor Biosynthesis

Small molecule primary metabolites are commonly leveraged as building blocks for the assembly of secondary metabolites; however, precursor deficiency can limit the productivity of natural product biosynthetic pathways [45] (Fig. 3a). Leveraging both native and engineered aTFs, several biosensor platforms have been developed for precursor detection as critical tools towards the long-term goals of optimizing metabolism and evolving *de novo* pathways for the production of natural products. For example, given the clinical significance of polyketides, there has been much interest in applying biosensor platforms to addressing long standing problems related to polyketide synthetic biology. Catalyzed by polyketide synthases (PKSs), polyketides are biosynthesized from the decarboxylative Claisen condensation of acyl-CoA building blocks. The potential modularity and versatility of such building blocks for accessing new-to-nature polyketides is driving development of biosensors that could be used to regulate their biosynthesis and to guide high-throughput engineering. Several aTFs utilize acyl-CoAs as effectors including AccR, which recognizes acetyl-, propionyl-, and methylcrotonyl-CoA [26], and FapR, which can detect its native malonyl-CoA (mCoA) as well as various C2-derivatives [46]. FapR-based biosensors have been used to produce oscillators that simultaneously regulate mCoA production via the upregulation of acetyl-CoA carboxylase and fatty acid biosynthesis [47]. Moreover, the newly identified promiscuity of this biosensor [46] could be leveraged for the directed evolution of *de novo* pathways to mCoA derivatives for precursor-directed biosynthesis of polyketide natural products.

Isoprenoids are derived from the isomeric precursors, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), to yield >80,000 unique compounds. Although no native transcription factor biosensor platforms have been identified for DMAPP, a fusion between isoprene synthase, IspS, and the DBD of AraC was developed as a DMAPP responsive regulator of the pBAD promoter [48]. Extending IspS applications, an isoprene biosensor was developed that allows monitoring the intracellular concentration of isoprene in single bacterial cells by modifying the TbuT transcription factor with a detection limit of 0.1 mM. The fluorescence signal of isoprene producing *E. coli* correlated to the amount of produced bioisoprene [49]. Isoprene biosensors could be leveraged for high-throughput screening of isoprenoid biosynthesis and potentially be used for the design of artificial hemiterpene biosynthetic pathways [50].

Dynamic Metabolic Control for Pathway Optimization

Metabolic engineering can alter pathway flux to optimize cellular processes for improved titers of relevant natural products. However, the resulting imbalance of stringently regulated metabolites often imposes inherent challenges including the production of toxic intermediates, reduced catalytic efficiency, and inhibition of downstream pathways. Dynamic pathway regulation can be implemented to mitigate these effects through pathway-specific biosensor-based strategies [51,52], pathway-independent circuits [53], or a combination of these strategies [54]. While metabolic engineering for pathway optimization has been well explored, the following examples demonstrate the importance of implementing biosensors for improved dynamic control.

Introducing the mevalonate (MVA) pathway into a heterologous host often results in a flux imbalance of the isoprenoid precursors (IPP) and farnesyl pyrophosphate (FPP), which are toxic upon accumulation [55]. Previously, the expression levels of the 'top' three genes in the MVA (MvaT) (Fig. 3b) pathway have been balanced using tunable intergenic regions (TIGR), increasing mevalonate production by 7-fold [56]. Thus, in order to balance the 'lower' part of the MVA pathway (MvaB) (Fig. 3b) and to increase the available FPP, a TIGR library was constructed and screened using an FPP sensor plasmid, in which Green Fluorescence Protein (GFP) was under the control of the *E. coli* rstA promoter [55]. The rstA promoter responds to the accumulation FPP to regulate the expression of zeaxanthin thus creating a crosslink between the cell's metabolic state and the pathway expression of the desired product [55]. To further reduce the metabolic burden, the TIGR optimized MvaB pathway plasmid and MvaT pathway were assembled on a single plasmid which resulted in a ~28% higher zeaxanthin production [55]. Furthermore, the PgadE/ IA44 sensor, which are downregulated by FPP and IPP respectively, were utilized to dynamically control the TIGR-mediated MVA pathway to prevent a toxic accumulation of precursors and further improve the zeaxanthin production by 1.6- and 1.7-fold [55].

Microbial production of fatty acid hydrocarbons, such as 1-alkene, which can be biosynthesized from activated fatty acids by polyketide synthases or from free fatty acids via cytochrome P450 enzymes, offer a sustainable opportunity for biofuels and olefins [57]. Recently, a *Saccharomyces cerevisiae* strain was engineered to improve the production of 1-alkenes by eliminating competing fatty acid consumption pathways and introducing the desaturase-like enzyme UndB from *Pseudomonas fluorescens* (PfUndB) [57]. These cumulative mutations resulted in up to ~29 fold-improvement of 1-alkene production [57]. To dynamically balance cell growth and product formation, PfUndB expression was regulated by the GAL7 promoter, which functions under low glucose levels, thereby enabling a distinct growth phase and a production phase that resulted in 100% higher 1-alkene titers [57]. Furthermore, FapR-based platforms have been used to dynamically control polyketide biosynthesis. For example, 6-methylsalicylic acid synthase (6-MSA) production capacity was increased 260% through the use of a hybrid yeast Prm1-FapR protein to regulate the malonyl-CoA dependent repression of fatty acid biosynthesis and the activation of the 6-MSA pathway genes, respectively [58].

Dynamic control has also been applied to improving titers via pathway independent regulatory elements, such as quorum-sensing (QS) circuits. A novel QS-based CRISPRi (EQCi) circuit, cell density-dependent gene regulation, was utilized in the rapamycin-producing strain *Streptomyces rapamycinicus* to autonomously and dynamically regulate multiple gene targets at once [33]. The EQCi circuit was designed to utilize the srbA promoter (srbAp) from *S. rapamycinicus*, which is under control of SrbR, to drive dCas9 expression [33]. At high cell density of *S. rapamycinicus*, the SrbR reaches a threshold in which it will no longer bind to srbAp, allowing for dCas9 expression, thereby switching the EQCi circuit on [33]. Furthermore, to improve rapamycin titers, three essential pathways were downregulated using this novel circuit resulting in the highest reported rapamycin titer of 1836 ±191 mg/L, an increase of ~660% compared to the wild-type producing strain [33]. Additionally, components from the *lux* and *esaR* QS systems were combined in *E. coli* to produce a single circuit to dynamically regulate a gene of interest [59]. This system was

applied to control the flux through the naringenin biosynthetic pathway and found that down-regulation of endogenous mCoA genes resulted in a 140% higher titer [59]

Biosensor-Guided Enhancements of Final Product Titers

Biosensors can also be utilized to circumvent screening bottlenecks for natural product pathway and enzyme engineering by providing a precise high-throughput tool for final product detection and titer quantification, e.g. erythritol, a secondary metabolite, using the engineered aTF, EryD [60]. This platform was coupled with automatic strain mutagenesis and screening to characterize more than 1152 strains in one week. The top producing engineered strain produced more than 148 g/L erythritol and is the first reported work to develop EryD as a high throughput biosensor.

Similarly, a rational circuit design has been coupled with selection to increase cellular tolerance to toxic products. The MexR transcriptional repressor, derepressed in the presence of pinene, was employed to regulate expression of the AcrAB-TolC efflux pump, which provides tolerance to toxic compounds, such as the terpene pinene, but can inhibit cell growth when overexpressed, thus creating a synthetic feedback loop [34]. Subsequently, a synthetic promoter library containing MexR binding sites was created to drive the expression of GFP [34]. In addition, the *mexA-mexR* binding sequence was isolated and incorporated into different regions of the library [34]. To add feedback, the variants from the promoter library were used to control the expression of the *acr*AB pump in varying levels of pinene [34]. In order to determine which MexR promoter class led to pinene tolerance and responded by turning on the efflux pumps, next generation sequencing was conducted post selection [34]. Under pinene treatment, there was a strong selective pressure for MexR binding sites that were immediately upstream of the *acrAB* gene in addition to a convergence of promoter sequences which suggested that their goal of using feedback control to balance pinene and pump toxicity was successful [34].

Biosensors for Detecting Natural Product Tailoring Modifications

Following the biosynthesis of the natural product core structure, enzymatic tailoring including phosphorylation, methylation, hydroxylation, and glycosylation, are frequently carried out to optimize bioactivity of the mature natural product [61]. Often, the remarkable flexibility of downstream enzymes that tailor natural product scaffolds, including methyltransferases (MTs), hydroxylases, and glycosyltransferases, enables molecular diversity. Highly specific engineered aTFs for a modified target compound could be leveraged to detect natural product tailoring steps which can then be targeted via a biosensor-guided approach to identify new biocatalysts or to evolve existing ones. The application of a vanillate biosensor for reporting the regioselective methylation of catechol exemplifies this vision. The VanR-VanO vanillate sensor system was engineered to selectively detect vanillate but no other methylated regioisomers or biosynthetic precursors [62]. Subsequently, the biosensor was used to identify the conversion from protocatechuate to vanillate catalyzed by prospective O-MTs. First, deletion of the methionine biosynthesis regulator MetJ improved the conversion of protocatechuate to vanillate by increasing the pool of the methyl donor, S-adenosylmethionine (SAM). Remarkably, using the biosensor,

three previously uncharacterized O-MTs were identified that supported the conversion of protocatechuate to vanillate [62]. As achieved in the vanillate example, a single aTF-based biosensor to report natural product tailoring needs to discriminate between the acceptor substrate and the tailored product. This could be done by leveraging the aforementioned strategies to engineer the required selectivity into a given aTF scaffold. Alternatively, a pair of ratiometric biosensors could be used for this purpose. For example, a FRET-based ratiometric sensor that incorporates the TetR repressor protein enabled correlation of fluorescence and tetracycline concentration allowing for accurate quantification enabling homogeneous assays without washing steps [63].

Although biosensors that directly detect the tailored product are likely preferred, aTFs with the required selectivity might not always be available. The development of biosensors that detect a cofactor, precursor, or byproduct of the tailoring reaction have also begun to be investigated for their potential utility. An example of this is the development of a genetically encoded ratiometric biosensor for NADH/NAD+ based on the redox-responsive transcription factor Rex [64]. The sensor was successfully deployed in a proof-of-principle high-throughput screen to enrich high NADH mutant strains that were diluted 10,000-fold in wild-type cells.

Conclusions and Future Outlook

Traditional engineering strategies for improving natural product titers and accessing non-natural designer compounds are often hampered by low-throughput screening technologies. Yet, the emergence and development of genetically-encoded biosensors have allowed for the rapid screening of potentially millions of variants to enhance pathway and enzymatic efficiencies. Subsequently, robust high-throughput screening platforms based on aTFs have enabled the detection of diverse natural product classes and their small molecule building blocks which has spurred the application of directed evolution and metabolic engineering of natural product biosynthetic pathways. We envision that these strategies will be particularly valuable to design aTFs with a defined linear range of detection in order to engineer prototype microbial strains for the biosynthesis of natural products, their analogues, and precursors.

Despite their utility, the discovery, characterization, and optimization of aTF-based biosensor platforms remains a critical bottleneck to their industrial application. aTF-based biosensors are limited in terms of their adaptability within host microbes to modulate proteins or complex gene networks. The transcription factors themselves must properly fold, maintain solubility *in vivo* and be amenable to engineering efforts. Indeed, effectors of transcription factors must be cell-wall permeable, readily available for binding, non-toxic, and stable inside the cell. Furthermore, it can be difficult to identify well-characterized aTFs that fit specific needs (e.g., specific effector or biosynthetic system). Even when a promising candidate aTF is identified, it can be difficult to identify a corresponding RBS and promoter system to make a functional gene circuit, given the diversity of microbial regulatory mechanisms [67]. However, as emerging technologies are developed, biosensor engineering is expected to progress towards more rapid, rational, and computational-based design for the facile development of highly targeted biosensors for a specific molecule of interest [66]. For

example, the use of cell-free technology allows for quick prototyping of gene circuits for *in vitro* detection of molecules, or for future *in vivo* biosensor operation.

Recent advances utilizing cell-free transcription-translation to rapidly characterize novel biosensor components and aTF effector promiscuity now offer prototyping capabilities to quickly overcome these challenges, specifically issues with effector permeability and toxicity [27,65]. The application of directed evolution to tailor the effector selectivity of aTFs continues to provide biosensors with exquisite detection capabilities. We expect future trends in biosensor design to apply new, open-access computational tools that enable rational engineering of the intricate conformational dynamics of aTFs via homology modeling and molecular docking, which would otherwise be arduous using traditional engineering strategies. Foremost, *de novo* protein design is set to provide customizable aTFs biosensors from the start that can be applied across diverse classes of molecules. The use of these tools has rapidly accelerated engineering efforts to enable the generation of smart libraries to effectively find active-site pockets or to simply generate highly targeted biosensors for a specific molecule of interest. Together, these technologies underlie the foundations of screening throughput and genetic diversity, which are at the forefront of evolutionary biosensor engineering. Herein, we highlight our vision for biosensor-guided approaches towards natural products and their derivatives to include directed evolution of aTFs, advances in cell-free methods to characterize them, new applications of computational tools, and the deployment of engineered aTF biosensors to address longstanding problems in natural product biocatalysis.

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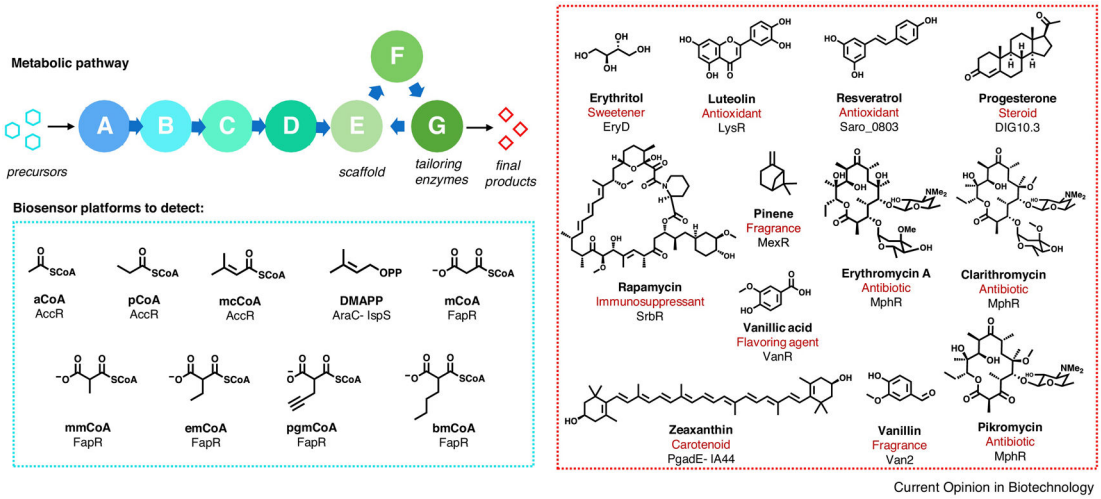

Figure 1.

Coupling genetically-encoded aTF biosensor platforms to natural product biosynthesis and engineering. Natural product biosynthetic logic is frequently divided into precursor generation, scaffold assembly, and tailoring, thereby providing several entry points for detection by aTFs. A variety of biosynthetic precursors (outlined in blue) and mature natural products (outlined in red), have been detected by aTF-based biosensors.

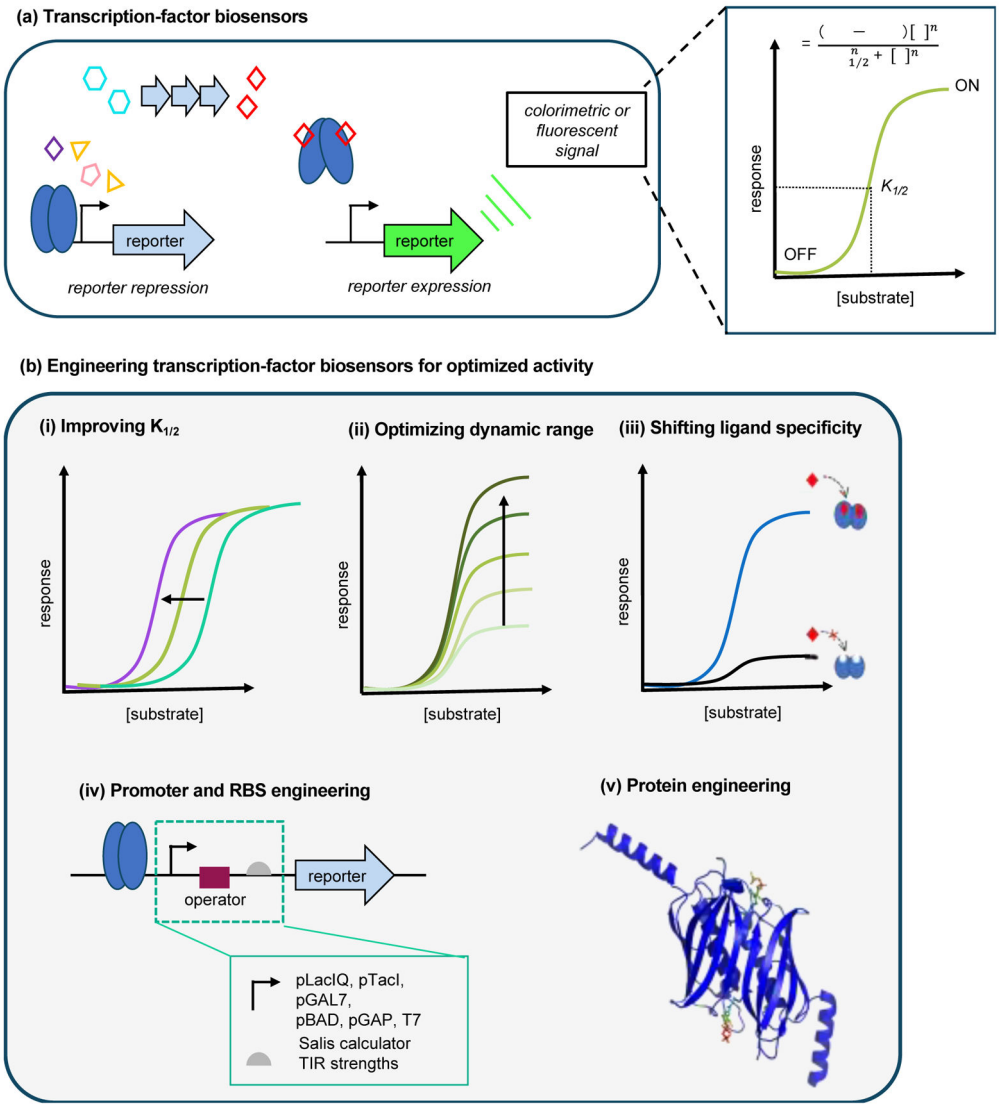

Figure 2.

Engineering and optimizing transcription factor-based biosensors. (a) Genetic circuit of a biosensor platform whereby the aTF regulates the expression of downstream genes by binding to its cognitive operator sequence. In the presence of an inducer, the system will derepress, allowing for the expression of a reporter signal. The corresponding dose response curve describing the response as a function of effector concentration is used to evaluate the biosensor transfer functions, as defined by the Hill equation. (b) aTF biosensor platforms can be tailored for a preferred function, including (i) sensitivity, (ii) dynamic range, (iii) effector specificity, (iv) control over transcription and translation via promoter and RBS engineering, (v) tailored substrate selectivity and/or promiscuity via protein engineering.

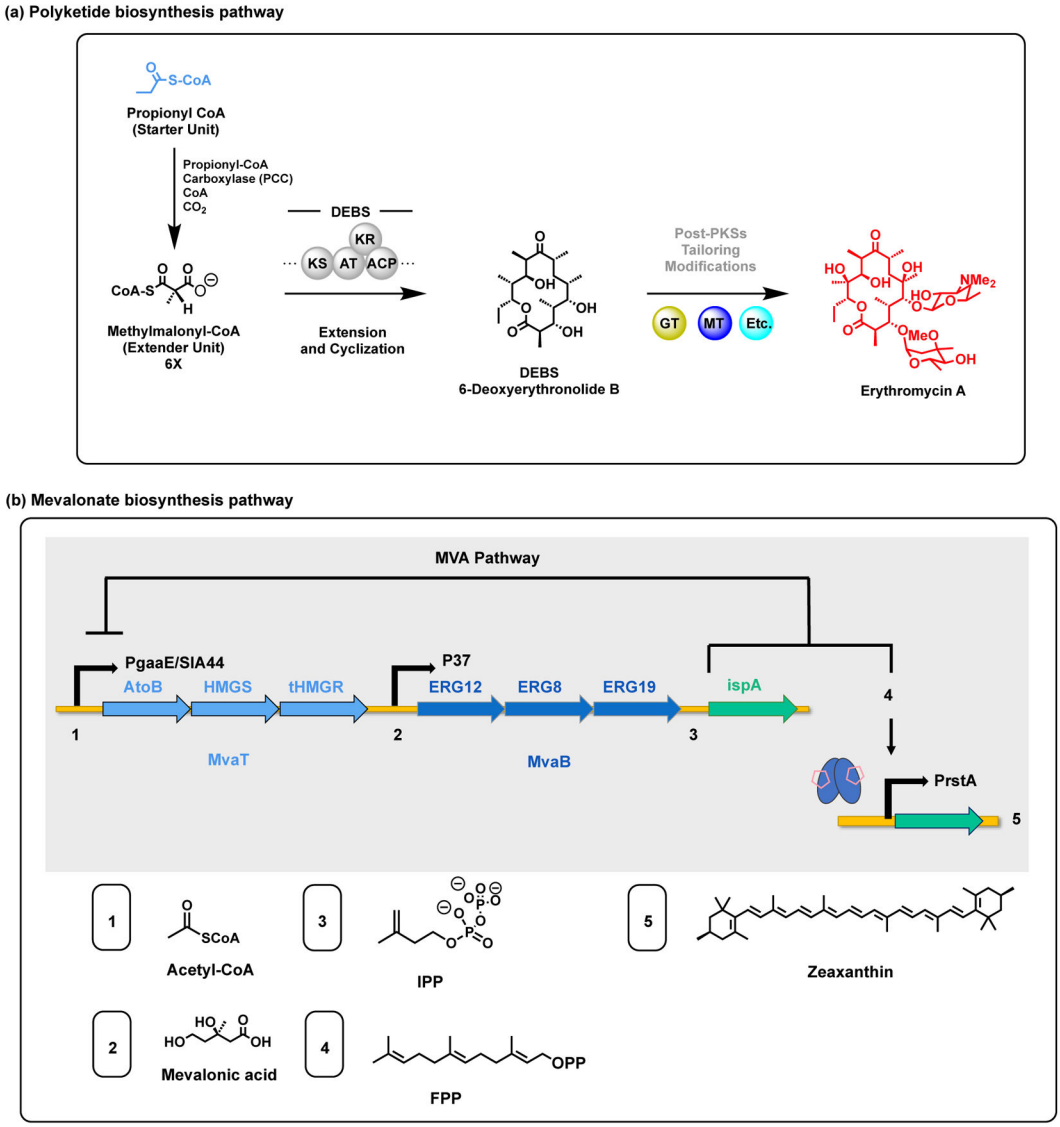

Figure 3.

Basic mechanism of (a) polyketide biosynthesis catalyzed by modular Type I PKSs and (b) the mevalonate pathway for hemiterpene production.

Table 1.

Examples of aTFs that respond to diverse natural products

aTF	EFFECTOR	NATURAL PRODUCT CLASS
HcaR	*Trans*-cinnamic acid [15]	Phenyl-propanoid; Used in manufacture of flavors, dyes and pharmaceuticals
ItcR	Itaconic acid [16]	Dicarboxylic acid; Building block for resins, paints, plastics and synthetic fibers
AraC	Ectoine [17], triacetic acid lactone [18], mevalonate [19]	Carboxamidine, polyketide byproduct, terpene precursor
BenM	*Cis,cis*-muconic acid [20]	Dicarboxylic acid; Used for the manufacture of plastics
FdeR	Naringenin [21]	Flavonoid
QdoR	Quercetin and kaempferol [21]	Flavonoid
NitR	Caprolactam [22]	Lactam; Used in the manufacture of plastics
AvaR1/AvaR2	Avenolide [23]	Avenolide is a signaling molecule for avermectin biosynthesis
JadR1/JadR2	Chloroamphenicol and jadomycin B [24]	Polyketides
ChnR	ε-caprolactam, δ-valerolactam, and butyrolactam [25]	Lactam biosynthesis
AccR	Acetyl-, propionyl, methylcrotonyl-, malonyl-, and methylmalonyl-CoA [26]	Extender units for the biosynthesis of fatty acids and Polyketides
MphR	Erythromycin, azithromycin, clarithromycin and roxithromycin [12,27]	Polyketides
TtgR	Naringen, phloretin and genistein [28]	Flavonoids
TetR	Tetracycline, simocyclinone D8, actinorhodin [29]	Tetracyclines
QacR	Vanillin [30]	Terpene
OtrR	Oxytetracycline [31]	Tetracycline
CtcS	Chlortetracycline [32]	Tetracycline
SrbR	Rapamycin [33]	Polyketide
MexR	Pinene [34]	Terpene