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Research review paper

Optogenetic tools for microbial synthetic biology

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ABSTRACT

Chemical induction is one of the most common modalities used to manipulate gene expression in living systems. However, chemical induction can be toxic or expensive that compromise the economic feasibility when it comes to industrial-scale synthetic biology applications. These complications have driven the pursuit of better induction systems. Optogenetics technique can be a solution as it not only enables dynamic control with unprecedented spatiotemporal precision but also is inexpensive and eco-friendlier. The optogenetic technique harnesses natural light-sensing modules that are genetically encodable and re-programmable in various hosts. By further engineering these modules to connect with the microbial regulatory machinery, gene expression and protein activity can be finely tuned simply through light irradiation. Recent works on applying optogenetics to microbial synthetic biology have yielded remarkable achievements. To further expand the usability of optogenetics, more optogenetic tools with greater portability that are compatible with different microbial hosts need to be developed. This review focuses on non-opsin optogenetic systems and the current state of optogenetic advancements in microbes, by showcasing the different designs and functions of optogenetic tools, followed by an insight into the optogenetic approaches used to circumvent challenges in synthetic biology.

1. Introduction

Synthetic biology is a burgeoning discipline that seeks to employ engineering principles for rational engineering and designing on the existing biological systems to create artificial capabilities that can solve real-world problems (Choi et al., 2019; Choi et al., 2020). Editing genetic blueprints and fine-tuning gene expressions (Yoo et al., 2020) are among the well-established modalities to tune various biological responses. With the advent of sophisticated gene-editing technologies such as recombinant DNA and CRISPR technologies (Choi and Lee, 2016; Wang et al., 2019), artificial components and functions can be integrated into microbes for biotechnological or biomedical applications. Most prominently, microbes have been engineered into sustainable cell factories capable of synthesising a wide variety of value-added chemicals and pharmaceutical compounds to meet the industrial and biomedical demands which are unmet by synthetic chemistry (Bailey, 1991; Davis et al., 2021; Keasling, 2010; Lee et al., 2012).

One of the important keys to ensuring the economic viability of these microbial cell factories, however, is the capability to finely and dynamically tune the endogenous and engineered pathways in order to minimize the trade-offs between the growth and productivity of microbial hosts (de Lorenzo et al., 2018; Ko et al., 2020). Traditionally, the strategy used to increase the yield of desired products has been only focused on the permanent modulation of genes (Holtz and Keasling, 2010). Although the technique has been somewhat effective, in many cases, it causes an imbalanced distribution of metabolic flux, thereby imposing a metabolic burden on the host, retarding the cellular growth, and jeopardising the overall production performance (Brockman and Prather, 2015; Holtz and Keasling, 2010; Jung et al., 2021; Liu et al., 2018a; Pouzet et al., 2020). Given these complications, rewiring the genetic network of microbial chassis through a more non-invasive and dynamic technique is highly desired to improve the biotechnological production profile, and reduce or even avoid the undesirable trade-offs.

The most common type of dynamic control in metabolic engineering has been a two-phase fermentation binary switch, which divides the growth and production phases using inducible promoters that can respond to different types of external stimuli such as chemical inducers, intracellular or extracellular metabolite concentrations, pH, temperature, and heat (Brockman and Prather, 2015; Lalwani et al., 2018; Liu et al., 2018b; Venayak et al., 2015). Despite these efforts, the pursuit for

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a dynamic control remains unmet because these stimuli often entail toxicity, poor cellular uptake of molecules, complicated pharmacokinetics, cross-reaction with undesired signalling pathways, and more importantly, most of these inductions often being irreversible.

2. Do it the light way – optogenetics

Over the last decade, a breakthrough in synthetic biology has made the long sought-after goal attainable. Underpinning by a technology known as optogenetics, light-inducible systems are constructed using genetic engineering and optical approaches to dynamically control

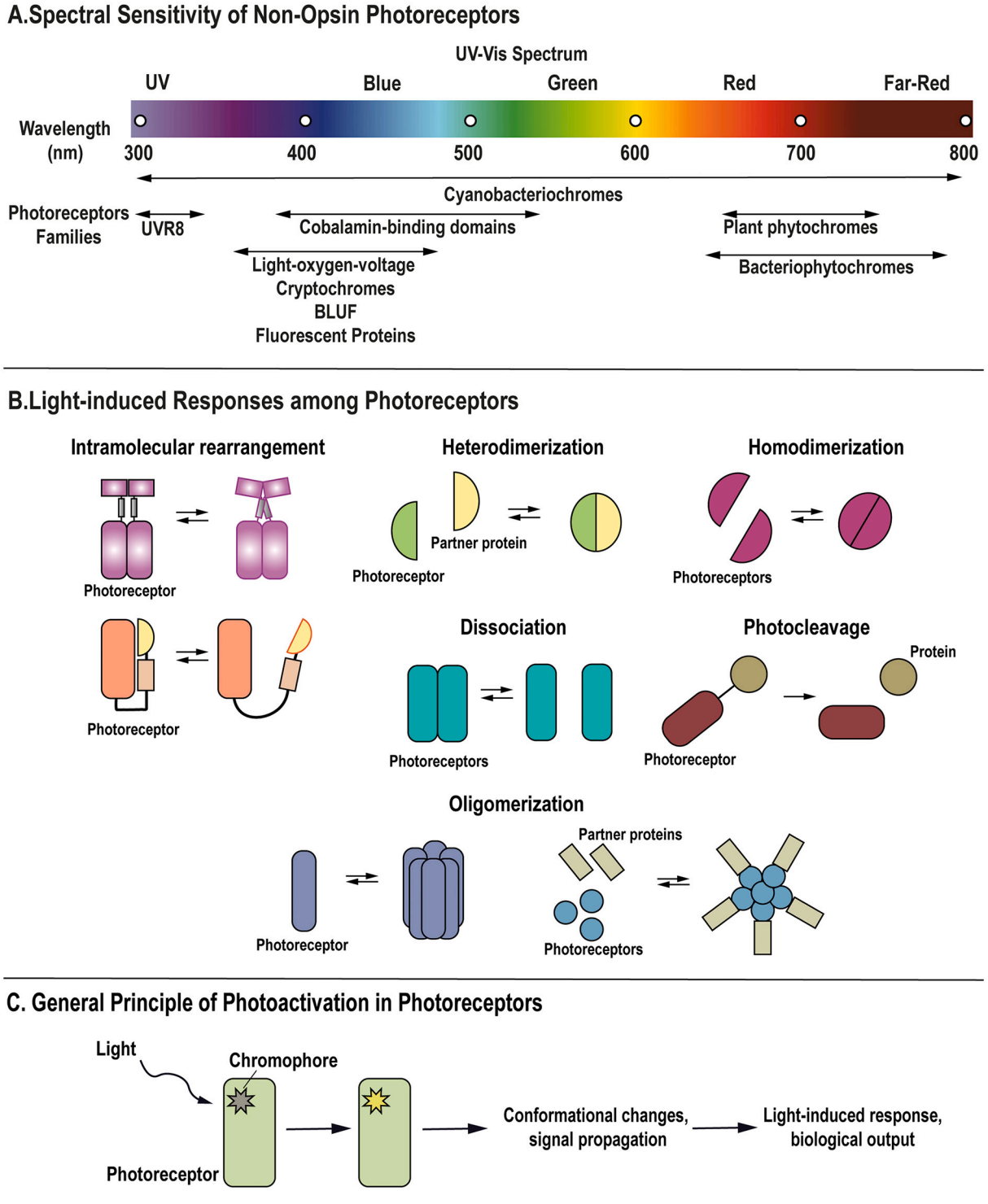

Fig. 1. Overview of non-opsins photoreceptors families and their activities. A. Different photoreceptors incorporate specific chromophore cofactors to perceive wavelengths that span across the UV-vis spectrum. B. Upon activation by light, photoreceptors (as illustrated in different geometry shapes for simplicity) display different types of light-inducible responses including intramolecular conformational changes, dimerization, oligomerization, dissociation, and photocleavage to carry out a wide range of biological activities. C. Generally, as the chromophore within the photoreceptor gets activated by light of specific wavelength, it triggers a series of conformational changes throughout the protein to initiate different types of responses (as shown in B.), along with the biological functions that are linked.

regulatory networks with explicit precision. Essentially, the technique employs light (of a certain wavelength from the UV-vis spectrum, (Fig. 1A) as the perturbation signal to manipulate responses in cells that have been genetically encoded with a light-sensing machinery. This relatively new technology emerged in the 2000s, with the term coined by a group of neuroscientists, Deisseroth and co-workers, who successfully demonstrated that the activity of neuron cells transfected with a microbial opsin (channelrhodopsin 2, which is a type of transmembrane light-gated ion channel found in nature), could be precisely controlled at spatiotemporal level upon induction of blue light (Bi et al., 2006; Boyden et al., 2005; Deisseroth et al., 2006).

Optogenetics offers distinctive advantages in terms of the use of light as the inducer (Baumschlager and Khammash, 2021). Light is an orthogonal stimulus and highly adjustable. By varying the focal point, intensity and frequency of the light exposure, it can achieve induction with high precision and in a spatiotemporal manner (Benzinger and Khammash, 2018; Boyden et al., 2005; Levskaya et al., 2005), thus enabling the selective interference of signalling pathways or biological processes. Unlike other stimuli such as chemical inducers, pH, temperature, or metabolites that are diffusible and can fluctuate with changing culture conditions, light provides greater control over the interference and is highly compatible with many systems, since it does not require transport protein to reach the photoreceptors, can be applied and removed at will, and leaves no chemical traces or requires the culture medium to be changed (Salinas et al., 2017). Hence, less cost and wastage are incurred. Moreover, in contrast to other light strategies that involve the incubation of chemically synthesised photosensitive compounds, optogenetics harnesses naturally evolved photosensory proteins that are genetically encodable in generally any living systems. Therefore, optogenetics is less invasive and well-suited for various *in vivo* applications beyond neuroscience (Boyden et al., 2005; Deisseroth, 2011; Guru et al., 2015).

Most optogenetics studies have been largely focusing on mammalian systems and biomedical applications using opsin photoreceptors. Opsin photoreceptors are membrane channel proteins that can be functionally divided into type I (ion channels) and type II (G-protein coupled receptors, GPCRs) opsins, where both associate with a retinal chromophore cofactor in order to perceive photons in the range of 400-600 nm (Duebel et al., 2015; Terakita, 2005; Yizhar et al., 2011). As they are naturally occurring light-modulated ion channels and GPCRs that govern the ion conductance and signalling across the cell membranes, they are preferentially exploited as tools or even therapies that can selectively intervene the neuronal activity or signalling pathways (Guru et al., 2015). As to microbes which are important workhorses for a wide range of biotechnological applications, the non-opsin photoreceptors such as Light-Oxygen-Voltage (LOV) domains, phytochromes, cyanobacteriochromes and cryptochromes are favoured due to their spectral diversity, modular topology, tunability and assorted molecular responses (Fig. 1B). These features open up many opportunities for optogenetic tools design and development to confer dynamic control around the central dogma of molecular biology.

Ever since the creation of bacteria that "sees" light and whose gene transcription is regulated in accordance to the flash of light (Levskaya et al., 2005), it has ushered in a new era in microbial synthetic biology, with many works exploiting the spatial and temporal capability of light for manipulation of regulatory pathways in cells, microbial physiology and metabolisms for useful applications. However, the realm of microbial optogenetics still remains in its infancy due to the lack of development in non-model microorganisms and compatibility for large-scale industrial applications (Liu et al., 2018a). This review aims to give an overview of the four major non-opsin photoreceptor families that are widely used in microbial synthetic biology, the design architectures and mechanisms of the established microbial optogenetic tools, followed by a highlight on the representative applications in synthetic biology for bioproduction, and then concluded by perspectives on the challenges and future directions of microbial optogenetics. The scope of opsin-based optogenetic tools and mammalian optogenetics will not be covered here as there are already numerous excellent reviews available (Kramer et al., 2021; Mansouri et al., 2019; Zhang et al., 2011).

3. Harnessing natural "light masters" for optogenetics

The photosensory proteins used in the optogenetic switches are mostly harvested from the photosynthetic organisms (Kolar et al., 2018). All kingdoms of life, including plants, algae, fungi, and bacteria, have naturally evolved with different families of photoreceptors to perceive and respond to light signals for survival and growth (Banerjee and Mitra, 2020; Gomelsky and Hoff, 2011; Shcherbakova et al., 2015).

Fundamentally, the light perception of natural photoreceptors is ascribed to an interacting chromophore cofactor that binds within their photosensory modules (Fig. 1A,C). Different chromophores cofactors absorb photons at different wavelengths spanning across the UV-vis spectrum, granting photoreceptors with differential association/dissociation wavelengths to carry out biological activities (Table 1) (van der Horst and Hellingwerf, 2004). Invariantly, the photocycle of all photoreceptors involves a series of conformational changes that bring about alteration of functions and dynamics upon light stimulation (Fig. 1C) (van der Horst and Hellingwerf, 2004; Ziegler and Moglich, 2015). Once the chromophore cofactor is activated by light, it initiates conformation rearrangement across the photosensory module and transduces a signal to its tethered effector module to elicit the associated light-dependent function. The photocycle event is fully reversible with most reversions being thermally driven, except for multichromatic photoreceptors in which their reversion can be catalysed through irradiation at a distinct wavelength (Camsund et al., 2011; Chernov et al., 2017; Rockwell and Lagarias, 2010).

Non-opsin photoreceptors are very diverse as they can be either transmembrane or cytosolic proteins that exhibit varying molecular outputs upon light stimulation (Fig. 1B), unlike opsin photoreceptors that are limited to membrane level control only. Furthermore, they are considered more versatile mainly because of their modular topology, in which the two functional moieties in the photosensory module – sensory and effector domains are situated in distinct proteins but their functionalities remain intact even when they are separated from each other (Möglich and Moffat, 2010). Such modularity is critical in order to allow the employment of various protein engineering techniques to create diverse optogenetic tools that can be linked with proteins of interest (POIs), and heterologously expressed in the desired microbial chassis to impart light-sensing machinery for functions (Di Ventura and Weber, 2021; Moglich et al., 2009a; Möglich and Moffat, 2010; Ziegler and Moglich, 2015).

3.1. LOV domains

The LOV domain is a small (~14 kDa) photosensory protein found in all kingdoms of life including plants, algae, diatom, fungi, and bacteria (Banerjee and Mitra, 2020; Kolar et al., 2018; Shcherbakova et al., 2015). The LOV photosensory domain absorbs blue light (440-450 nm) via flavins-based chromophores such as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) or riboflavin, which are ubiquitously available in all living systems (Losi et al., 2018). Given that the LOV domain is a subclass of the Per-ARNT-Sim (PAS) domain superfamily, whose members function as sensors of a wide range of environmental processes throughout biology (Taylor and Zhulin, 1999), they are commonly found to associate with a plethora of regulatory effector proteins such as kinases, phosphodiesterases, DNA-binding domains, and stress σ factors (Crosson et al., 2003). The photochemistry of LOV domain typically involves the formation of a thioether adduct between the C4 atom in the flavin chromophore and a conserved cysteine residue of the apoprotein, as well as a hydrogen-bonding rearrangement in the LOV core to transduce signal to the effector for downstream physiological roles (Glantz et al., 2016; Moglich et al., 2009b; Salomon et al.,

Table 1
Microbial optogenetic toolbox.

	Optogenetic tools/ systems	Photoreceptor and components	Source organism	Chromophore	ON/OFF stimuli	Host	Mode of action	Dynamic range (-fold)	References
GeneTranscriptionGene Transcription		***Two-component systems***							
	–	Cph8(Cph1-EnvZ)/OmpR	*Synechocystis* sp.	PCB	Dark/Red	*E. coli*	Intramolecular shift, photoactivated histidine kinase activity	Cph8-OmpR v2.0: 72	(Levskaya et al., 2005; Schmidl et al., 2014)
	pDawn pDusk	YF1(YtvA-FixL)/FixJ, cI/ P_R YF1(YtvA-FixL)/FixJ	*Bradyrhizobium japonicum*	FMN	Dark/Blue	*E. coli*	Intramolecular shift, photoactivated histidine kinase activity	Induction: 460 Repression: 70	(Moglich et al., 2009a; Ohlendorf et al., 2012)
	pDawn-Ag43	YF1(YtvA-FixL)/FixJ, cI/ P_R	*Bradyrhizobium japonicum*	FMN	Blue/Dark	*E. coli*	Intramolecular shift, photoactivated histidine kinase activity	15	(Jin and Riedel-Kruse, 2018)
	pDawn-Tn7	YF1(YtvA-FixL)/FixJ, cI/ P_R	*Bradyrhizobium japonicum*	FMN	Blue/Dark	*Pseudomonas aeruginosa*	Intramolecular shift, photoactivated histidine kinase activity	20	(Pu et al., 2018)
	pDawn	YF1(YtvA-FixL)/FixJ, cI/ P_R	*Bradyrhizobium japonicum*	FMN	Blue/ Dark/	*Vibrio natriegens*	Intramolecular shift, photoactivated histidine kinase activity	Not specified	(Tschirhart et al., 2019)
	–	CcaS/CcaR	*Synechocystis* sp.	PCB	Green/Red	*Synechocystis sp.*	Intramolecular shift, photoactivated histidine kinase activity	40	(Abe et al., 2014)
	–	CcaS/CcaR	*Synechocystis* sp.	PCB	Green/Red	*E. coli*	Intramolecular shift, photoactivated histidine kinase activity	CcaSR v1.0: 6 CcaSR v2.0: 117 CcaSR v3.0: 600	(Ong and Tabor, 2018; Schmidl et al., 2014)
	–	CcaS/CcaR	*Nostoc punctiforme*	PCB	Green/Red	*E. coli*	Intramolecular shift, photoactivated histidine kinase activity	Not specified	(Sugie et al., 2016)
	–	CcaS/CcaR	*Synechocystis* sp.	PCB	Green/Red	*Bacillus subtilis*	Intramolecular shift, photoactivated histidine kinase activity	*B. subtilis* CcaSR v0.2: >70	(Castillo-Hair et al., 2019)
	–	UirS/UirR	*Synechocystis* sp.	PCB	UV/Green	*E. coli*	Intramolecular shift, photoactivated histidine kinase activity	~ 6	(Ramakrishnan and Tabor, 2016)
	–	BphP1/PpsR2	*Rhodopseudomonas palustris*	BV	Far-red/ Red	*E. coli*	Intramolecular shift, dissociation, heterodimerization	< 3	(Ong et al., 2018)
		One-component systems							
	EL222 bidirectional system	EL222	*Erythrobacter litoralis*	FMN	Blue/Dark	*E. coli*	Homodimerization	Induction: 5 Repression: >3	(Jayaraman et al., 2016)
	-	EL222	*Erythrobacter litoralis*	FMN	Blue/Dark	*E. coli*	Homodimerization	>10	(Jayaraman et al., 2018)
	-	EL222	*Erythrobacter litoralis*	FMN	Blue/Dark	*Sinorhizobium meliloti*	Homodimerization	4	(Pirhanov et al., 2021)
	pBctrl	EL222	*Erythrobacter litoralis*	FMN	Blue/Dark	*Pichia pastoris*	Homodimerization	>79	(Wang et al., 2022b)
	LightOff, Inverted-LightOff systems	LEVI (LexA-VVD), cI/ $P_{\lambda(R-O12)}$	*Neurospora crassa*	FAD	Blue/Dark	*E. coli*	Homodimerization	Induction: >10000 Repression: >1000	(Chen et al., 2016)
	yLightOn	LVADO(LexA-VVD-Gal4AD)	*Neurospora crassa*	FAD	Blue/Dark	Yeast	Homodimerization	>500	(Xu et al., 2018)
	eLightON	LexRO (RsLOV-$LexA_{408}$)	*Rhodobacter sphaeroides*	FMN	Blue/Dark	*E. coli*	Dissociation	>500	(Li et al., 2020)
	paT7P-1	VVD-T7 RNAP	*Neurospora crassa*	FAD	Blue/Dark	*E. coli*	Homodimerization	62	

(*continued on next page*)

Table 1 (*continued*)

	Optogenetic tools/ systems	Photoreceptor and components	Source organism	Chromophore	ON/OFF stimuli	Host	Mode of action	Dynamic range (-fold)	References
									(Baumschlager et al., 2017; Han et al., 2017)
	LANS	AsLOV2, NLS, NES, LexA, Gal4AD	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	21	(Lerner et al., 2018; Yumerefendi et al., 2015)
	LINXa4	AsLOV2, NLS, NES, LexA, Gal4AD	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	15	(Yumerefendi et al., 2016)
	LINuS	AsLOV2, NLS, CRISPRi, Mxi1	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	10	(Geller et al., 2019)
	CLASP	LANS (AsLOV2, NES, NLS) + SynTF + LOVTRAP (AsLOV2, Zdk1)	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, dissociation, and uncaging	>20	(Chen et al., 2020)
	BLADE	VVD-AraC-DBD	*Neurospora crassa*	FAD	Blue/Dark	*E. coli*	Homodimerization	<7	(Romano et al., 2021)
	iLight	IsPadC BphP, $LexA_{408}$	*Idiomarina sp.*	BV	Far-red/ Red	*E. coli*	Tetramerization	115	(Kaberniuk et al., 2021)
	Two-hybrid systems								
	–	PhyB(NT)-Gal4/PIF3-GAD	*Arabidopsis thaliana*	PCB	Red/Far-red	Yeast	Heterodimerization	>1000	(Shimizu-Sato et al., 2002)
	–	PhyA-GalBD/FHL-AD, PhyA-GalBD/FHY1-AD	*Arabidopsis thaliana*	PCB	Red/Far-red	Yeast	Heterodimerization	~150, ~300	(Sorokina et al., 2009)
	–	Gal4BD-CRY2/Gal4AD-CIB1	*Arabidopsis thaliana*	FAD	Blue/Dark	Yeast	Heterodimerization	Not Specified	(Hughes et al., 2012)
	–	LexA-CRY2PHR/VP16-CIBN	*Arabidopsis thaliana*	FAD	Blue/Dark	Yeast	Heterodimerization	32	(Hughes et al., 2012)
	FUN-LOV	WC-1-Gal4BD/VVD-Gal4AD	*Neurospora crassa*	FAD	Blue/Dark	Yeast	Heterodimerization	>1300	(Salinas et al., 2018)
	PhiReX	PhyB-synTALE-DBD/ PIF3-VP64AD	*Arabidopsis thaliana*	PCB	Red/Far-red	Yeast	Heterodimerization	PhiReX1.0: 11 PhiReX1.1: 41	(Hochrein et al., 2017)
	Opto-T7RNAP*(563)	pMag/nMag, T7 RNAP	*Neurospora crassa*	FAD	Blue/Dark	*E. coli*	Heterodimerization	~300	(Baumschlager et al., 2017)
	yOTK	ZBD-CRY2/ CIB1-VP16	*Arabidopsis thaliana*	FAD	Blue/Dark	Yeast	Heterodimerization	~11	(An-Adirekkun et al., 2020)
Gene Translation	–	PAL, 04.17 RNA aptamer	*Nakamurella multipartita*	FMN	Blue/Dark	*E. coli*	Unknown	~10	(Weber et al., 2019)
	L-SCRaMbLE	PhyB(NT)/PIF3, Cre recombinases	*Arabidopsis thaliana*	PCB	Red/Far-red	Yeast	Heterodimerization	<180	(Hochrein et al., 2018)
DNA Modification	Opto-Cre-Vvd/Cre-Mag/Flp-VVD/Flp-Mag	Magnets, VVDs, Cre and Flp recombinase	*Neurospora crassa*	FAD	Blue/Dark	*E. coli*	Homodimerization	Not Specified	(Sheets et al., 2020)
	–	PhyB/(APB)PIF3, VMA inteins	*Arabidopsis thaliana*	PCB	Red/Far-red	Yeast	Heterodimerization	20	(Tyszkiewicz and Muir, 2008)
	Photosensitive degron (psd) module	AtLOV2, cODC1	*Arabidopsis thaliana*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	>7	(Renicke et al., 2013)
Post-Translational Modification	LinXa4-Bre1	AsLOV2, NLS, NES, chromatin modifier Bre1	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	Not specified	(Lerner et al., 2018)
	–	$AsLOV2^{iLID\ \Delta A146}$-cODC1	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	~40	(Hasenjager et al., 2019)
Second Messenger	BphS	BphG1-Slr1143	*Rhodobacter sphaeroides*	BV	Near-Infrared/ Dark	*E. coli*	Intramolecular shift, photoactivated enzyme	>50 (c-di-GMP level) ~40 (gene expression)	(Ryu and Gomelsky, 2014)
	LOV2GIVe	AsLOV2, GBA motif	*Avena sativa*	FMN	Blue/Dark	Yeast	Intramolecular shift, uncaging	Not Specified	(Garcia-Marcos et al., 2020)

Abbreviations: BV, Biliverdin Ixα; FAD, Flavin adenine dinucleotide; FMN, Flavin mononucleotide; PCB, Phycocyanobilin.

2000). Owing to their small size, the ubiquitously available associating chromophore cofactor, and diversity in the mechanisms of action, the photoreceptors from the LOV domain family have contributed to most of the currently available one-component optogenetic tools.

3.2. Cryptochromes

Cryptochrome 2 (CRY2) is a photolyase-like photoreceptor that detects blue light via FAD chromophore found in *Arabidopsis thaliana*, where they modulate photoperiodic flowering by associating with a cryptochrome-interacting basic-helix-loop-helix 1 (CIB1) protein in a blue light-dependent manner (Liu et al., 2008; Yu et al., 2010). Structurally, it is composed of a N-terminal photolyase homology region (PHR) domain that incorporates the FAD chromophore, and an intrinsically disordered yet functionally indispensable CRY C-terminal extension (CCE) domain that serves as the effector to display physiological events upon activation (Wang et al., 2017; Yang et al., 2001). While an explicit CRY2 photochemistry remains elusive (Losi et al., 2018; Yu et al., 2010), it is proposed that blue light induces phosphorylation at the CCE domain and alters the overall electrostatic interaction in the CRY2 protein, which then causes it to undergo conformational change to interact with other proteins for downstream function (Duan et al., 2017; Wang et al., 2018; Yu et al., 2007).

3.3. Phytochromes

Photosensory proteins from the phytochromes superfamily perceive photons in the red to near-infrared (NIR) range (620 – 800 nm) that has higher light penetration depth. Both plant phytochromes and cyanobacterial phytochromes utilize phycocyanobilin (PCB) as chromophore that is not natively available in non-photosynthetic organisms; whereas bacteriophytochromes utilize biliverdin Ixα (BV), that can be synthesized from heme in mammalian cells (Baumschlager and Khammash, 2021; Oliinyk et al., 2017). Phytochromes from disparate origins constitute a head-to-head dimer structure (Auldridge and Forest, 2011; Burgie et al., 2014) and they can be grouped into three subfamilies based on the topology and number of domains in their photosensory module, with a cGMP phosphodiesterase-adenylate cyclase-FhlA (GAF) domain being conserved among them as that is essential for chromophore binding and light perception (Oliinyk et al., 2017; Wiltbank and Kehoe, 2019).

The ability of phytochromes to photoconvert between red-light absorbing state (Pr) and far-red light-absorbing state (Pfr) is an intriguing feature. The intricate molecular basis of photoconversion among phytochromes has been reviewed in detail elsewhere (Burgie and Vierstra, 2014; Moglich et al., 2010; Oliinyk et al., 2017). Importantly, it involves a Z/E isomerization in the chromophore that provokes a cascade of conformational change throughout the photosensory protein to convert from Pr to Pfr conformer state and activate the downstream effector domains for diverse functions such as second messenger signalling, gene expression and regulation of transcription factors (Legris et al., 2019; Moglich et al., 2010). Furthermore, phytochromes have the tendency to either act as a short- or long-lasting light switch since they can revert to their ground state (Pr) within seconds catalysed by far-red light or, slowly with a timescale of hours in the dark (Burgie et al., 2014).

3.4. Cyanobacteriochromes (CBCRs)

Cyanobacteria is a class of photosynthetic prokaryotes that encodes for photoreceptors that are similar to phytochromes but have a substantially different and more diverse spectral characteristics from them (Wiltbank and Kehoe, 2019). They are known as cyanobacteriochromes (CBCRs) which encompass only a single knotless GAF domain to associate with a PCB or phycoviobilin (PVB) chromophore in the N-terminal photosensory module (Ikeuchi and Ishizuka, 2008). They differ from the phytochromes in terms of the photoconversion mechanism, where additional molecular interactions and conformational changes are involved to facilitate their signal transduction and downstream responses (Hirose et al., 2013; Ikeuchi and Ishizuka, 2008; Oliinyk et al., 2017). CBCRs are primarily found with a histidine kinase output domain that is associated with physiological activities such as chromatic acclimation (e.g. photoreceptor CcaS) and phototaxis (e.g. photoreceptor UirS) via a two-component signal relay mechanism (Auldridge and Forest, 2011; Mullineaux, 2001; Song et al., 2011; Wiltbank and Kehoe, 2019).

4. Microbial optogenetic toolbox

While opsins invariantly exist and transduce signal to control membrane channel opening through a single-component system (Deisseroth, 2011; Terakita, 2005), different non-opsin photoreceptors regulate biological processes such as gene expression, DNA modification, post-translational modification and second messenger signalling via a variety of mechanisms, which includes intramolecular conformational changes, dimerization, oligomerization, dissociation and photocleavage (Fig. 1B, Table 1) (Kolar et al., 2018). Depending on the photoreceptor property and mechanism of action, some of them require one, two or more components to complement the light signal transduction system. Alternatively, the natural photoreceptors can be reengineered to confer distinct modes of action in order to customize them for specific applications (Guntas et al., 2015; Strickland et al., 2012). Recent microbial optogenetic advancements are mostly demonstrated in model microbial chassis - *Saccharomyces cerevisiae* (yeast) and *Escherichia coli*, with only a very limited number of optogenetic systems have been expressed and tested in other model or non-model microorganisms (Table 1). To provide insights into the developed microbial optogenetic tools (Table 1), they are presented in the following based on their design architectures and that will be in terms of one-component system, two-component system and two-hybrid system accordingly.

4.1. One-component system

In one-component systems, the photosensory and effector domains are encoded in one same DNA sequence, resulting in a single protein (Yizhar et al., 2011). Such system architecture is more readily found in photoreceptors from the LOV domain family and therefore they are easier to work with from a genetic engineering perspective. Photoreceptors or optogenetic tools that operate in such system architecture also tend to be miniatured in size and have a faster ON/OFF kinetics than those requiring more than one component (Liu et al., 2018a) as they do not have to relay the signal over a long distance to the corresponding effector or response regulator. On top of that, they provide straightforward control over a particular biological event in cells by directly modulating the activity of target protein that has been appended to the effector domain of the photoreceptor. Once the photoreceptor is activated by light, a cascade of conformational changes will be transduced to the effector domain that exerts an allosteric or steric regulation at the effector domain, thus giving rise to different mechanisms of action such as uncaging, plasma membrane recruitment, enzyme activation, homodimerization, tetramerization, and dissociation (Fig. 1C and 2) (Baumschlager and Khammash, 2021).

4.1.1. Light-induced conformational change and uncaging

Plant-derived LOV2 domain is the most classic example of a single-component optogenetic system. They are found in *Avena sativa* phototropin 1 (AsLOV2) and *Arabidopsis thaliana* (AtLOV2), exhibiting a well-defined LOV2 photosensory core connecting to a C-terminal amphiphilic Jα-helix that docks against it during the ground state (Halavaty and Moffat, 2007). When triggered by blue light, a cascade of structural rearrangement will be initiated which then causes the Jα-helix to unwind and undock from the LOV β-sheet surface (Harper et al., 2004;

Harper et al., 2003). The photocycle occurs at a very fast rate in AsLOV2, where it restores its ground state structure at the timescale of seconds to minutes upon the withdrawal of blue light (Harper et al., 2003; Salomon et al., 2000).

To take advantage of this light-modulated LOV-Jα interaction which is also known as photocaging and uncaging, a protein of interest (POI) is appended at the end of the Jα-helix to impose a steric hindrance that deactivates the protein function during the ground state in the dark (Pudasaini et al., 2015). As the Jα-helix moves away from the LOV core upon photoexcitation, the protein regains its function or the ability to interact with other partner proteins (Fig. 2A). However, it is noteworthy that the protein fusion at the Jα-helix end has been reported to destabilize the LOV-Jα interaction which gives rise to basal activity in the dark (Garcia-Marcos et al., 2020; Strickland et al., 2008; Strickland et al., 2010). Moreover, together with the rapid photocycle of AsLOV2, these issues will diminish the dynamic range as well as the performance of optogenetic control in the host. For that, further optimization can be done through directed mutagenesis to stabilize the LOV-Jα docking in the dark (Strickland et al., 2010) or relocating the position POI at the Jα-helix for tighter photocaging effect in the dark (Garcia-Marcos et al., 2020; Lungu et al., 2012).

The optogenetic application of AsLOV2 has been very prominent in yeast system to perturb various regulatory functions, including gene expression, post-translational modification, and cell destiny, by controlling the nuclear translocation of regulatory proteins (POIs) (Table 1). In general, these optogenetic constructs are coupled with a nuclear localization signal sequence, such as an import or export signal so that the abundance of POIs in the nucleus, as well as the corresponding nuclear signalling events can be manipulated through AsLOV2 photocaging activity in response to blue light (Fig. 2A; Table 1) (Geller et al., 2019; Lerner et al., 2020; Lerner et al., 2018; Niopek et al., 2014; Niopek et al., 2016; Yumerefendi et al., 2015; Yumerefendi et al., 2016). In order to reduce leakiness of the nuclear signalling activity, the manipulation of protein abundance in the nucleus can also be improved by incorporating an additional optogenetic system to export and anchor the proteins to a specific site such as mitochondria and plasma membrane (Chen et al., 2020; Yumerefendi et al., 2016). For instance, Chen and coworkers have developed a controllable light-activated shuttling and plasma membrane sequestration (CLASP) system (Chen et al., 2020), which is composed of two previously developed AsLOV2-based optogenetic systems – light-inducible nuclear shuttle (LANS) (Yumerefendi et al., 2015) and LOV2 Trap and Release of Protein (LOVTRAP) (Wang et al., 2016) (Fig. 2B). LOVTRAP localizes the entire protein construct to the plasma membrane because of the LOV2-Zdk binding affinity in the dark, but as the photoinduced intramolecular conformational change occurs, the LANS module dissociates from the anchor site and carries the target protein along with it into the nucleus to exert its function (Fig. 2B) (Chen et al., 2020). Alternatively, the subcellular protein abundance can also be regulated by appending the LOV2 scaffold with a photosensitive degron (psd) module for proteasomal degradation of the entire fusion protein (Renicke et al., 2013; Usherenko et al., 2014).

Although it is described earlier that light-regulated transmembrane signalling is dominated by opsin photoreceptors, a group of scientists has taken the advantage of AsLOV2 as a single-component system to manipulate GPCR signalling cascade by incorporating a short sequence from the Gα-Binding-and-Activating (GBA) motif to the Jα-helix scaffold for heterotrimeric G-protein activation (Garcia-Marcos et al., 2020). This construct not only bypasses the chromophore availability and receptor desensitization issues that are often associated with opsin photoreceptors (Garcia-Marcos et al., 2020), but more importantly, this work further exemplifies the versatility of non-opsin photoreceptors and their unlimited possibilities through modular designs.

4.1.2. Light-induced conformational change and membrane recruitment

The LOV domain from the fungus *Botrytis cinerea* BcLOV4 (Fig. 2C) is a newly discovered photoreceptor that has functions associated with GPCR signalling pathway by regulating the recruitment regulator of G protein signalling (RGS) domains (Glantz et al., 2018). Upon light activation, BcLOV4 also demonstrates a similar LOV-Jα undocking response, but as it consists of an additional polybasic amphiphilic α-helix extended from the Jα-helix, it can be recruited to the plasma membrane through an electrostatic protein-lipid interaction with diffusion-like kinetics and very rapid photocycle at the timescale of seconds (Fig. 2C) (Glantz et al., 2018). This novel single-component membrane recruitment system is a valuable addition to the optogenetic toolbox, as the existing membrane recruiting systems such as LOVTRAP (Fig. 2B) requires the engineering of an anchor protein at the specific site and a small molecule with distinct binding affinity to the photoreceptor in ground and active states (Guntas et al., 2015; Strickland et al., 2012; Wang et al., 2016). Moreover, a recent study has reported that BcLOV4 is a dual-sensor as it responds to both heat and blue light, which can be utilized as multi-state controller in synthetic biology (Benman et al., 2022; Wilson, 2022).

4.1.3. Light-induced homodimerization

The EL222 photoreceptor protein from *Erythrobacter litoralis,* and Vivid (VVD) photoreceptor protein from fungus *Neurospora crassa* are both members of the LOV domain photoreceptor family that respond to blue light stimulation and they share similar photoinduced response involving the exposure of an interaction surface for homodimerization. As many transcription factors operate in the form of dimers (Amoutzias et al., 2008; Thliveris et al., 1991), EL222 and VVD are exploited extensively in the regulation of gene transcription through homodimerization (Table 1).

In particular, the EL222 is a naturally evolved transcription factor that comprises a N-terminal LOV photosensory domain, and a C-terminal helix-turn-helix (HTH) DNA binding domain (Rivera-Cancel et al., 2012; Zoltowski et al., 2013). In dark condition, EL222 manifests an inhibitory contact between the 4α-helix motif and the LOV surface, which prevents DNA binding (Fig. 2G) (Nash et al., 2011). When exposed to blue light, conformational rearrangement occurs and that abolishes the inhibitory interaction, allowing dimerization between HTH domains and binding to the specific EL222-binding motif in the promoter region for transcription (Zoltowski et al., 2013). The application of EL222 for gene regulation simply requires the incorporation of the EL222-binding motif in the promoter of choice because of its natural function as transcription activator. The promoter can be engineered to be either inductive or repressive by adjusting the position of EL222-binding motif along the promoter region to be away or that overlaps with the RNA polymerase binding sequence (Jayaraman et al., 2016). Owing to the simplicity and compactness of the EL222 optogenetic system, both the inductive and repressive EL222 systems can even be expressed in parallel within a single cell to achieve bidirectional gene regulation (Jayaraman et al., 2016). The EL222 gene regulation system has been adopted in different industrially important microbial hosts including *E. coli*, yeast, and very recently in *Pichia pastoris* with minimal leakiness (Table 1 and 2) (Ding et al., 2020; Reshetnikov et al., 2022; Wang et al., 2022a; Zhao et al., 2018).

In addition, attributing to the fast photocycle kinetics and ability to be restimulated multiple times after recovering to its ground state (Motta-Mena et al., 2014), EL222 can afford pulsative light stimulation to finely regulate gene expression in a dose-dependent manner (Benzinger and Khammash, 2018). Especially in the context of real industrial-scale applications, this property is vital for attaining a truly dynamic control over biological processes in cell or cell-free systems (Jayaraman et al., 2018), or even as tools or models to study the stochastic process of gene expression (Benzinger and Khammash, 2018; Chen et al., 2020; Rullan et al., 2018).

The use of VVDs in yeast has been validated with robust performance (Table 1) (Chen et al., 2016; Xu et al., 2018). The structure of VVD consists of a N-terminal α-helical cap (Ncap) that docks against the core of LOV domain in its dark-adapted state (Zoltowski et al., 2007). Similar

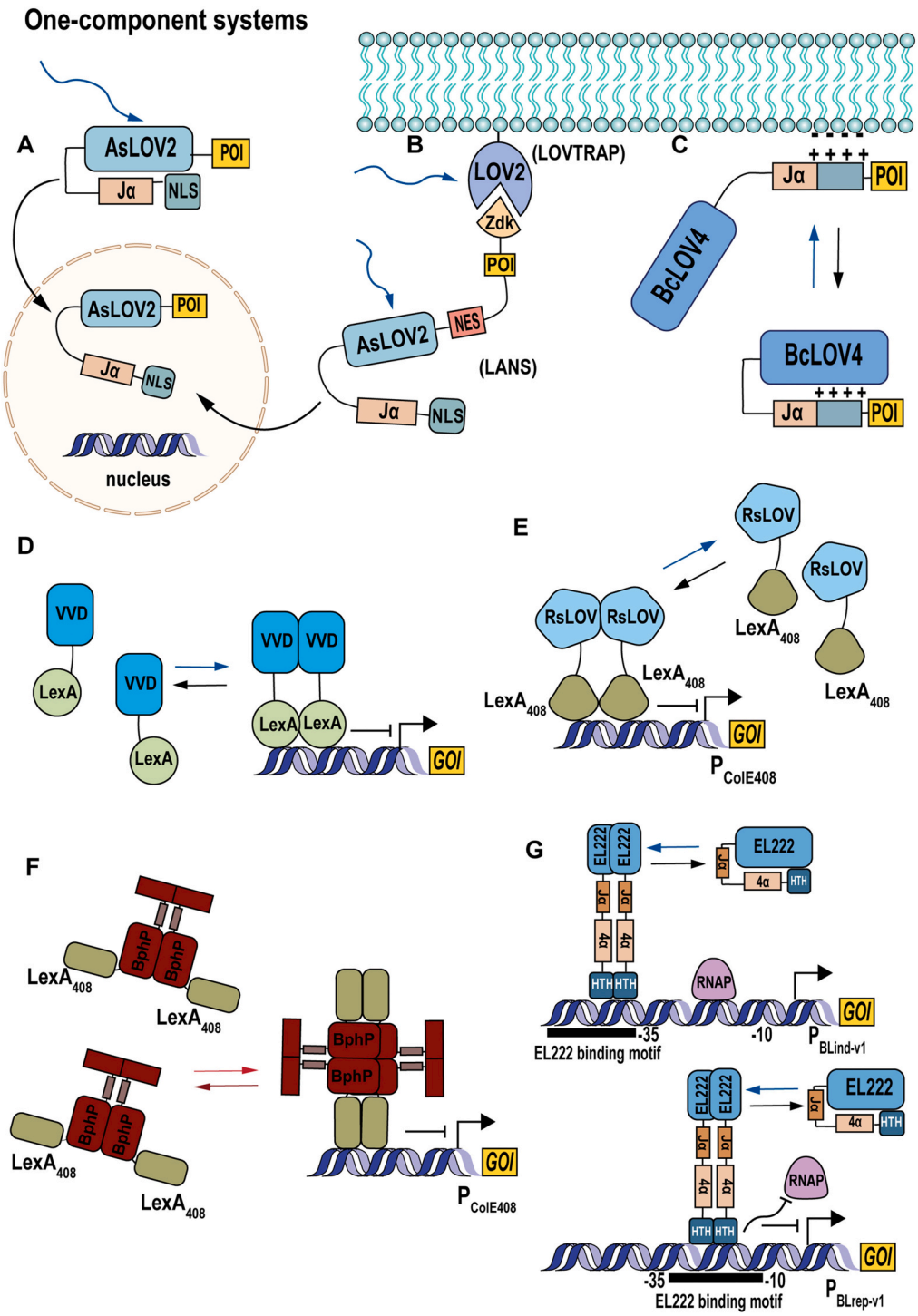

Fig. 2. Representative one-component optogenetic systems with distinct modes of action. A-E and G are blue-light optogenetic systems while F is a red-light optogenetic system. A. A nuclear localization signal (NLS) that is masked by the LOV-Jα interaction is exposed under blue light (450 nm) to translocate the protein of interest (POI) into nucleus for function. B. The CLASP system is constituted of two AsLOV2 constructs to regulate protein localization and activity. In the dark, the entire construct is bound to the LOV domain that is anchored to the plasma membrane (LOVTRAP). When induction of blue light occurs, both LOV2s undergo conformational changes that result in dissociation of Zdk protein from the anchored LOV domain and photo-uncaging of NLS, which then leads to nuclear transportation of the POI for downstream function. The constitutive nuclear export signal (NES) appended in between the photosensory domain of AsLOV and the POI will export the entire construct from nucleus to bind with the anchored LOV domain again when the illumination ceases (LANS, light-activated nuclear shuttle). C. The polybasic helix of BcLOV4 is uncaged upon blue light induction and translocates to the plasma membrane through electrostatic interaction. D. Vivid (VVD) which homodimerizes under blue irradiation controls the oligomer state and activation of transcriptional repressor, LexA during gene regulation. E. Light-induced function of RsLOV is similar to VVD, but it dissociates under blue light and homodimerizes in the dark. F. For the iLight system, the engineered bacteriophytochrome photoreceptor (BphP) is conjugated with mutated repressor, $LexA_{408}$ that gets activated upon tetramerization of the photoreceptor under the illumination of red light (660 nm) and dissociates under near-infrared light (780 nm) to regulate gene expression. G. EL222 is a transcription factor that homodimerizes under blue light to initiate or inhibit gene expression depending on the placement of DNA binding motif on the promoter and whether that interferes with the RNA polymerase activity. Note: B, F and G are modified from (Chen et al., 2020), (Kaberniuk et al., 2021) and (Jayaraman et al., 2016) accordingly.

Table 2
Applications of optogenetic systems in microbial synthetic biology.

Host	Optogenetic system	Photoreceptor(s) and components	Associated/ dissociated stimuli	Notes	References
Yeast	Opto-EXP; Opto-INVRT	VP16-EL222	Blue/Dark	Periodic light pulses protocol can replenish the intracellular level of NAD^+ to avoid metabolic arrest and upregulate the production yields.	(Zhao et al., 2018)
	Opto-INVRT7	VP16-EL222	Blue/Dark	An optimized version of Opto-INVRT by re-engineering the yeast galactose regulon to create a high-performance Super GAL1 Promoter ($P_{GAL1\text{-}S}$) with a high induction power (133-fold), and control the protein abundance via degradation tags or modules to improve activation kinetics by 7-fold.	(Zhao et al., 2020)
	OptoAMP	$VP16\text{-}EL222^{A79Q}$	Blue/Dark	Prolonged EL222 half live gives a more sustained activation state, requiring less light for activation. The use of different periodic light pulses during the proposed three phase fermentation scheme (growth, induction, production) can maximize production.	(Zhao et al., 2021)
	OptoCluster PixELL	Cry2-VioC, Cry2-VioE; PixD-VioC, PixE-VioE	Blue/Dark	Compartmentalization of metabolic enzymes enhances the efficiency of metabolic channelling and selectively drives metabolic flux into the desired production pathway to reduce formation of unwanted by-products.	(Zhao et al., 2019)
E. coli	RGB system	Cph8/OmpR, CcaS/CcaR, YF1/FixJ, CRISPR/dCas9	Red, Green, Blue	A multiplexed gene control system involving three sgRNA cassettes targeting different enzyme genes, with each being individually regulated by red-, green- and blue-responsive two-component systems to alter acetate production without compromising cell growth.	(Fernandez-Rodriguez et al., 2017)
	–	CcaS/CcaR	Green/Red	Controls the flux ratio of EMP and oxPP pathways at between 50:49 and 0.5:99 to regulate the intracellular supply of NADPH.	(Tandar et al., 2019)
	–	CcaS/CcaR	Green/Red	Reduces the accumulation of MGO, which exhibits cytotoxicity, by approximately 68%.	(Senoo et al., 2019)
	–	BphS	NIR/Dark	*E. coli* is engineered to express both BphS and tryptophan synthase (TrpBA) from *Salmonella enterica* for NIR-regulated biofilm formation and indole-tryptophan biotransformation.	(Hu et al., 2019)
	–	PhyB/PIF3, VMA intein, T7 RNAP	Red, IPTG	Requires induction of both IPTG and red light to uncouple the fermentation from cell growth to lycopene production phase. The induction is irreversible.	(Raghavan et al., 2020)
	OptoLAC	YF1/FixJ (pDawn system)	Blue/Dark	Replaces the signal specificity of *lac* operons from IPTG to blue light.	(Lalwani et al., 2021)
	Opto-CRISPRi	EL222, CRISPR/dCas12a	Blue/Dark	A multiplexed gene control system using crRNA arrays that contain multiple competing pathway genes in tandem to synergistically block competing pathways during production phase in response to blue light.	(Wu et al., 2021)

Abbreviations: crRNA, CRISPR RNA; IPTG, Isopropyl β-D-1-thiogalactopyranoside; EMP, Embden-Meyerhof-Parnas; MGO, methylglyoxal; NAD^+, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NIR, Near Infra-red; oxPP, oxidative pentose phosphate; sgRNA, single guide RNA.

to EL222, light-induced structural reorientation removes the steric hindrance at the LOV core, thus exposing the interface for LOV-LOV dimerization between two VVDs (Zoltowski and Crane, 2008). To employ VVDs, each of them is fused with identical POIs to enable reconstitution as homodimers and activate the protein function in a blue light-specific manner. Based on this principle, a very robust (>10 000-fold repression) light-regulated gene expression system has been constructed (Fig. 2D) (Chen et al., 2016). On the other hand, VVDs can also be utilized to transform non-light-responsive homodimers in cells to act in response to blue light by replacing their native dimerization domains with that of VVDs. For instance, the endogenous arabinose (AraC) in *E. coli* was engineered in such a way, thereby making its activation and gene transcription from its native promoter P_{BAD} inducible by blue light (blue light-inducible AraC dimers in *Escherichia coli* (BLADE)) (Romano et al., 2021).

4.1.4. Light-induced dissociation

Alternatively, light can trigger dissociation to sequester a biological function or event too. For example, the RsLOV photoreceptor from the photosynthetic gram-negative bacterium *Rhodobacter sphaeroides* ATCC17025 inherently exist as homodimer in dark circumstances (Conrad et al., 2013; Hendrischk et al., 2009). When coupled with transcription activators, it functions as a light-repressive transcriptional system as the RsLOV dimers dissociate into monomers under blue light. Such system activates gene expression in the dark and can be beneficial in certain cases especially for limited light settings. Alternatively, RsLOV can still serve as a light-inducible system by coupling with a transcriptional repressor such as LexA that is found endogenously in *E. coli*, so that light-induced dissociation will uplift the repression on the gene expression, and promote transcription instead (Fig. 2E) (Li et al., 2020). To prevent crosstalk and off-target effects on LexA-regulated pathways in the host, engineered promoter or mutated LexA ($LexA_{408}$) can be integrated instead (Li et al., 2020).

4.1.5. Light-induced tetramerization

The tetramerization mechanism is an example of synthetic light-induced response that is entirely borne out of the construction of a synthetic photoreceptor (Kaberniuk et al., 2021). All the optogenetic constructs acting through one-component system design that has been discussed so far only respond to blue light using the scaffold of various types of LOV photosensory proteins as the basis. For broader scope of *in vivo* applications, light options that have a higher degree of cell penetration, and lower energy to avoid the introduction of phototoxicity under prolonged exposure to light is highly desirable. A single-component, NIR-responsive optogenetic gene regulation system, iLight was devised to address these limitations (Kaberniuk et al., 2021). Using the *Idiomarina* sp. A28L-derived bacteriophytochrome, IsPadC (Gourinchas et al., 2017) as scaffold, its photosensory core module is isolated and fused with the DNA-binding domain of $LexA_{408}$. As the result of this engineering, iLight propagates the light signal through a novel dimer-to-tetramer structural mechanism which then leads to the homodimerization of the appended transcription repressor for gene regulation (Fig. 2F) (Kaberniuk et al., 2021). However, the underlying mechanism that contributes to the function of the optogenetic construct still remains poorly understood.

4.1.6. Light-induced enzymatic activation

Unlike the majority of the phytochromes that encode for histidine kinases, the bacteriophytochrome BphG1 that derived from *Rhodobacter sphaeroides* bears a GGDEF-EAL effector domain, which encodes for a diguanylate cyclase activity to regulate of intracellular cyclic dimeric GMP (c-di-GMP) second messenger in response to NIR light (712nm)

(Auldridge and Forest, 2011; Tarutina et al., 2006). Given that c-di-GMP signalling pathway accounts for many bacterial physiology and behaviour (Ryu et al., 2017), a research group has engineered a potent hybrid photoreceptor, BphS by harvesting the photosensory domain from BphG1 and the GGDEF domain from *Synechocystis* sp. Slr1143 to control the intracellular c-di-GMP level and its downstream signalling pathway in *E. coli* (Ryu and Gomelsky, 2014). However, a phosphodiesterase is also needed in order to regulate the c-di-GMP level in cells. Hence, in the study, a phosphodiesterase protein, YhjH was coupled to the BphS system to regulate the intracellular c-di-GMP level and a downstream MrkH transcription factor for gene regulation in the bacteria (Ryu and Gomelsky, 2014). This system has since been featured in numerous microbial studies to manipulate their physiology and the microbial consortia via c-di-GMP pathways (Huang et al., 2018; O'Neal et al., 2017; Ryu et al., 2017).

4.1.7. Light-induced RNA binding

The existing optogenetic tools were not able to provide direct interrogation at the translation level until the recent discovery of a natural RNA hairpin-binding photoreceptor which confers a light-regulated PAL-RNA interaction (Weber et al., 2019). Structurally, it encompasses a PAS, a AmiR and NasR Transcription Antitermination Regulators (ANTAR), and a LOV domain, therefore it is named PAL photoreceptor (Weber et al., 2019). PAL is a constitutive dimer with an uncanonical C-terminal LOV photosensory and N-terminal effector architecture. In the study, the blue light-activated PAL photoreceptor

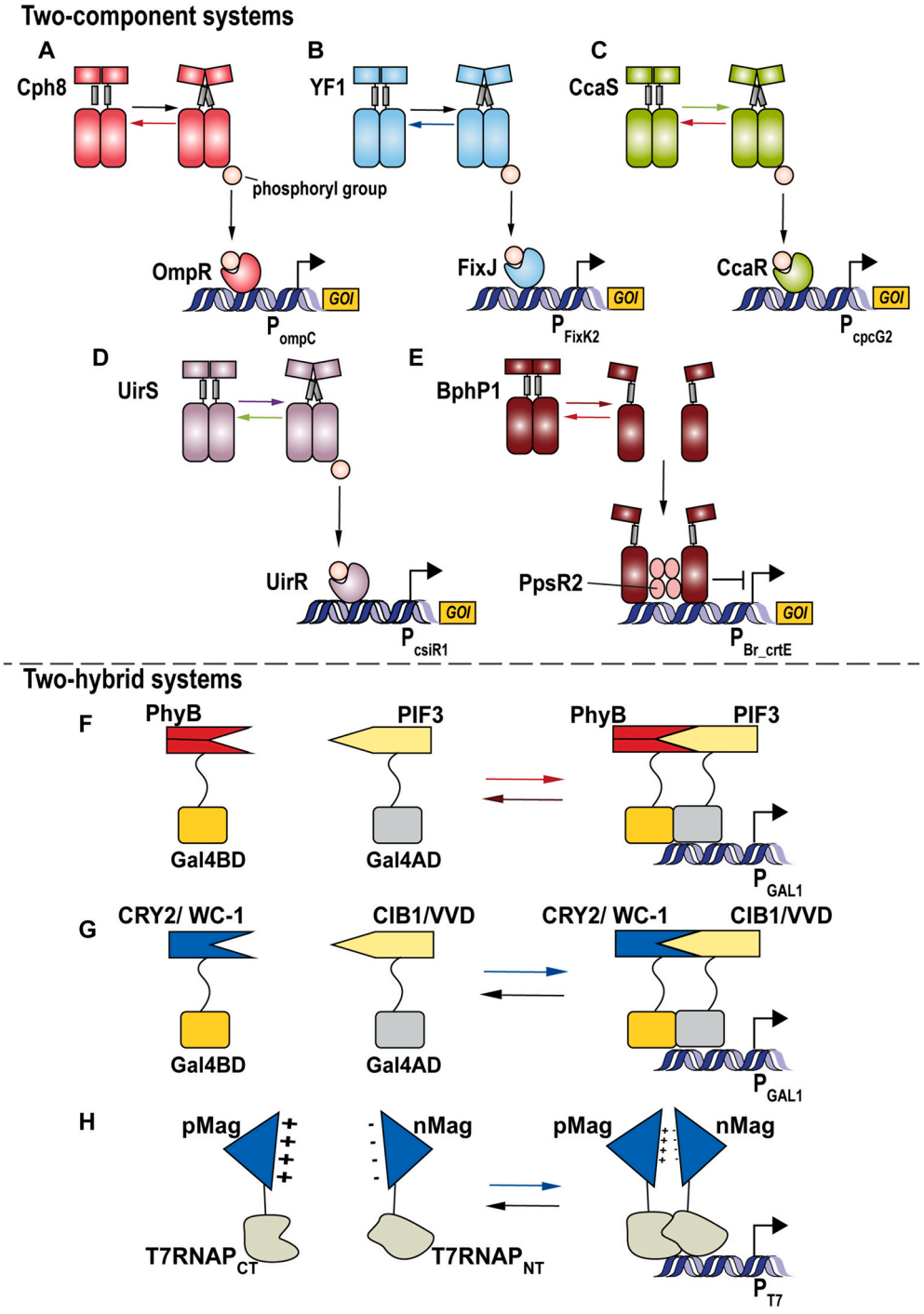

Fig. 3. Optogenetic switches shown are constructed based on a two-component system or a two-hybrid system. A-E. Light-regulated two-component systems. A. Cph8 is the engineered photoreceptor that reacts upon the illumination of red light (650 nm). In the dark, the photoreceptor Cph8 is activated and undergoes autophosphorylation, followed by transferring a phosphoryl group to its response regulator, OmpR to activate it for initiation of transcription from the cognate promoter, P_{ompC}. The same applies to B. When illuminated with red or blue (470 nm) light, the photoreceptor dephosphorylates and returns to its ground state, which in turn represses the gene transcription. Same working principle applies to C, D but they are regulated by green (535 nm)/red (672 nm), and purple (405 nm)/green (534 nm) lights respectively. E. BphP1 photoreceptor dissociates into monomers upon stimulation of near infrared light (760 nm) and binds to its response regulator PpsR2 to repress gene transcription from the promoter P_{Br_crtE}. Upon illumination of red light (680 nm) it reverts back to its ground state. Two-hybrid systems are designed based on binding affinity of photoreceptors and their interacting proteins for heterodimerization and they are usually employed to reconstitute non-identical protein fragments such as Gal4 binding and activation domains (Gal4BD and Gal4AD) or split T7 RNA polymerase (T7RNAP) proteins to control gene expression, as shown in F-H.

bound to a PAL-specific aptamer sequence that was placed upstream of the ribosome binding site of a reporter gene to attenuate the translation in *E. coli* (Weber et al., 2019). This represents a novel approach to perturbate RNA-mediated physiological processes, for which the authors termed as optoribogenetics (Weber et al., 2019).

4.2. Two-component system

The two-component system (TCS) is a multistep signal transduction pathway that is widespread in bacteria (Parkinson, 1993; Stock et al., 2000). Classically, TCSs can capture various environmental stimuli via a transmembrane histidine kinase protein, which then transfers the signal to its native response regulator through a phosphorylation mechanism, and that subsequently initiates transcription from the cognate output promoter (Fig. 3A-D) (Ma et al., 2017). Most phytochromes and cyanobacteriochromes contain a clear histidine kinase output domain that is associable to TCS, most extant light-responsive TCS is produced utilising photosensory modules generated from them (Burgie and Vierstra, 2014; Krall and Reed, 2000; Wiltbank and Kehoe, 2019). Given that the overall architecture of photoreceptors and TCS are modular, a TCS optogenetic system can be constructed by repurposing the existing TCS in the host and engineered to become light-responsive through sensory domain swapping, or by directly integrating the host with a naturally occurring photosensitive TCS that has been assembled with a gene of interest (GOI) at its output promoter for optogenetic regulation (Möglich and Moffat, 2010). However, the availability of BV or PCB cofactor is the main issue when assembling a light-regulated TCS in the target host. Though this can be overcome via exogenous supplementation or incorporation of the cofactor biosynthesis genes (Gambetta and Lagarias, 2001; Ge et al., 2013; Mukougawa et al., 2006; Uda et al., 2020), this adds an additional layer of complexity for application as it could be an issue when importing the system into hosts with limited capacity.

4.2.1. Light-induced activation of histidine kinase activity

The Cph8/OmpR system (Fig. 3) was the first optogenetic gene regulation system engineered from a light-responsive and a natural non-light-responsive TCS. Cyanobacterial phytochrome 1 (Cph1) is a PCB-dependent, red light-controlled histidine kinase found in cyanobacterium *Synechocystis sp.* PCC6803 (*Synechocystis* thereafter) (Yeh et al., 1997). The EnvZ/OmpR TCS system in *E. coli* originally regulates the membrane porin expression in response to environmental osmolality, but it was then reprogrammed to respond to red light stimulation by swapping the osmotic sensor in the EnvZ histidine kinase with the photosensor from Cph1(Levskaya et al., 2005). The hybrid construct Cph8 (Cph1-EnvZ, Fig. 3A) was then employed to control the expression of *LacZ* reporter that encoded for a black beta-galactosidase pigment. Under the projection of spatially patterned light, black pigments produced on the agar plate formed a monochrome image that directly corresponded to the light projection image, thus it was regarded to act just like a biological photograph (Levskaya et al., 2005).

Following that, a similar notion was utilised to create another hybrid, YF1 (LOV_{YtvA}-FixL) using the FixL histidine kinase from *Bradyrhizobium japonicum* and the LOV photosensory domain of YtvA from *Bacillus subtilis* to alter the signal specificity from oxygen to blue light (Losi et al., 2002; Moglich et al., 2009a). Ultimately, the YF1/FixJ optogenetic control system (also known as pDusk, Fig. 3B) was able to induce gene expression by up to 70-fold in the dark relative to blue light condition (Table 1) (Ohlendorf et al., 2012). On the other hand, the signal polarity can also be inverted to become inducible under blue light (pDawn) by incorporating a λ phage cI repressor at the downstream of promoter P_{FixK2}, and a LacI repressor that is placed downstream of the cI-responsive promoter P_R to turn on the GOI when irradiated with blue light (Ohlendorf et al., 2012).

Naturally occurring light-responsive TCSs that are found in *Synechocystis* include the photoreceptor CcaS and its response regulator, CcaR that respond to response to green and red lights (Fig. 3C), and also the most blue-shifted photoreceptor UirS and its response regulator UirR that can be stimulated by UV and green lights (Fig. 3D) (Hirose et al., 2008; Ramakrishnan and Tabor, 2016; Wiltbank and Kehoe, 2019). They both are often directly integrated into target hosts to manipulate gene expression by placing the GOI downstream the specific cognate promoters of the photoreceptors. BphP1 is a bathy bacteriophytochrome in *Rhodopseudomonas palustris* that exhibits an inverted photocycle (Pfr to Pr) and regulates gene expression in response to NIR light (Ong et al., 2018; Rottwinkel et al., 2010). Unlike the rest, BphP1 activates its response regulator PpsR2, which is a transcriptional repressor, through protein-protein interaction in order to regulate gene transcription via its cognate promoter, P_{Br_crtE} (Fig. 3E) (Ong et al., 2018). Though BphP1/PpsR2 system can adapt to light fluctuations in minute-timescale, its biological relevance is limited by its short dynamic range of 2.5-fold due to the strong background activity under dark condition (Ong et al., 2018). It is thought that the tendency of PpsR2 to undergo oligomerization is the contributing factor to the low efficacy of the system and therefore adopting a truncated version, QPAS1, with no oligomerized interaction domains, could potentially be more advantageous (Kichuk et al., 2021; Redchuk et al., 2018).

The spectrum diversity encoded by phytochromes and CBCRs that provides potential for multiplexed platforms to achieve multichromatic control of gene expression, is the most significant advantage of these optogenetic TCSs. As most of them incorporate the same chromophore for signal initiation but have different spectrum specificities and outputs, combining several TCSs into a system to allow multiplexed gene regulation is possible.

The idea has been realized in *E. coli* co-expressed with both CcaS/CcaR and Cph8/OmpR which share the common chromophore (PCB) to control the expression of *lacZ* simultaneously and produce bacterial photography that corresponds with the projection of a green-red composite image (Tabor et al., 2011). In addition, considering the convenience of photoreceptor YF1 that relies on an endogenously available chromophore, it is incorporated alongside CcaS/CcaR and Cph8/OmpR to generate a RGB system in *E. coli* that is connected to a resource-allocation system to manipulate the production of three coloured pigments (red, green and blue) for generating coloured photographs on the bacterial cultured plates (Fernandez-Rodriguez et al., 2017). Furthermore, the authors also demonstrated the interplay between optogenetic and CRISPR interference (CRISPRi) techniques (Qi et al., 2013) to dynamically and precisely regulate gene expression. CRISPRi technique involves the use of a catalytically dead Cas9 mutant (dCas9) which forms a complex with a single guide RNA (sgRNA) consisting of a site-specific DNA sequence (normally 20-nt) for target site binding and a handle for forming the specific RNA secondary structure. As the dCas9 lacks nuclease activity, it only binds to the targeted DNA region to block the binding and/or movement of RNAP, causing full-length mRNA unable to be synthesized and thus, transcription is inhibited (Tong et al., 2020). In the RGB system, three sgRNAs are constructed to carry *pta, ackA* and *poxB* genes from the acetate metabolic pathway respectively. The transcription of these sgRNAs is then regulated by the blue-, green- and red- inducible promoters independently to optimize the flow of flux and minimize the trade-offs between growth and production of acetate (Fernandez-Rodriguez et al., 2017). More recently, a CcaS/CcaR-YF1/FixJ has been devised as a dual-switch to dynamically regulate polyhydroxybutyrate production in *E. coli* (Wang et al., 2022b).

4.3. Two-hybrid system or split proteins system

Two-hybrid system is another routinely used tactic to design and construct light-inducible systems. The design rationale of this system essentially harnesses the intrinsic or synthetic binding affinity between the photoreceptor along with its cognate interacting protein to confer the heterodimerization interaction. The pair of photoreceptor and its interacting partner will be appended with POIs, which give rise to two hybrid proteins that act like a 'bait' and 'prey' for the recruitment and

reconstitution of proteins or split protein fragments (Bruckner et al., 2009). A special feature of this system is the ability to reconstitute non-identical proteins, as this would be suboptimal or even not feasible due to incorrect orientation if homodimerized photoreceptors pairs were used. Furthermore, a potential caveat of this system lies within the photoreceptor pairs that are mainly sourced from the plant kingdom which are relatively large in size and difficult to be heterologously expressed, especially in bacteria (Deforce et al., 1991; Mukougawa et al., 2006; Senturk et al., 2020). Perhaps due to the lack of proficiency in post-translational modification among bacteria that impedes optimal functional expression, most of the currently available light-inducible heterodimerization systems are applied exclusively in eukaryotes (Table 1) (Kichuk et al., 2021; Kolar et al., 2018). On the other hand, synthetic heterodimer pairs can also be engineered using the LOV domain scaffold (see LOVTRAP in Section 4.1.1).

4.3.1. *Light-induced heterodimerization*

The heterodimerization interaction between proteins has been exploited either as a photoswitch that exerts a direct control over a biological event by turning on the protein function upon binding or, an indirect control by translocating the proteins away from the site of action to sequester the event. A range of regulatory proteins, especially the transcription factors, recombinases, or enzymatic proteins have been controlled by such mode of action (Table 1).

In *A. thaliana*, phytochrome B (PhyB) regulates gene transcription by forming complexes with different types of phytochrome-interacting factors (PIFs) in response to red or far-red lights. PhyB is found residing in the cytoplasm in its ground state (Pr), but when triggered by red light it converts to the active state (Pfr) and translocates into the nucleus to dimerize with PIF3 or PIF6 to form a regulatory complex for gene expression (Pham et al., 2018; Van Buskirk et al., 2012). The property of PhyB/PIFs was harnessed to bring the first demonstration of a genetically encoded light-induced system that designed based on the generic yeast two-hybrid system logic (Fig. 3F) (Shimizu-Sato et al., 2002). The N-terminals of PhyB and PIF3 are each fused with a Gal4 DNA-binding domain (PhyB-GBD) and a Gal4 activation domain (PIF3-GAD) respectively, so that as the PhyB and PIF3 dimerizes under red light, they reconstitute as a functional transcription factor for gene expression (Fig. 3F). Other photoreceptor pairs such as PhyB/PIF6, PhyA/FHY1 and PhyA/FHL have also been engineered in a similar manner (Fig. 3F, Table 1) (Pathak et al., 2014; Sorokina et al., 2009).

However, a comparative study has suggested that the PhyB/PIF3, rather than the PhyB/PIF6 optogenetic system has better feasibility and effectiveness for gene regulation in yeast (Pathak et al., 2014). Interestingly, the employment of another *A. thaliana* derived photoreceptor, PhyA, and its interacting partners FHY1 (far-red elongated hypocotyl 1) or FHL (FHY1 like protein) for photoinducible gene expression systems has not only been reported with superior induction rates than that of the PhyB/PIFs (Table 1), but also a surprisingly superior induction rate in the absence of PCB supplementation in the culture, implying the presence of an unidentified chromophore compound that is naturally available in yeast (Sorokina et al., 2009).

The association between CRY2 and its cognate partner, CIB1 under blue light has also been harnesses to regulate gene expression (Fig. 3G, Table 1) (Hughes et al., 2012). As the light-sensing of CRY2 is mediated by a FAD cofactor which is ubiquitous in bacteria and yeast, the application does not require the additional supplementation of chromophore cofactor like in the case of phytochromes and CBCRs. In general, the CRY2/CIB1 system is attractive in terms of its high sensitivity to light induction, strong activation under blue light pulses, rapid ON/OF kinetics with binding dissociation taking about 10 minutes, and with minimal concern of baseline activity in the dark (Kennedy et al., 2010; Pathak et al., 2014). Nevertheless, its broad usability is limited by its large size and suboptimal expression profile (Kennedy et al., 2010). Whilst using a truncated $CRY2_{PHR}$ variant would improve the expression level in cells, this also constitutes a considerably higher background interaction with CIB1 or CIBN under dark circumstances (Kennedy et al., 2010). For that, several authors have made optimizations to reduce the protein size, improve their dynamic range and even create mutants with fast and slow kinetics (Pathak et al., 2014; Taslimi et al., 2016).

It is worth noting that CRY2 also exhibits clustering property to form photobodies in plants (Mas et al., 2000; Ozkan-Dagliyan et al., 2013). Further exploration on the clustering behaviour has revealed that the oligomerization (CRY2-CRY2) (Fig. 1B) and heterodimerization (CRY2-CIB1) can occur concomitantly through distinct interacting sites (Bugaj et al., 2013; Che et al., 2015). However, CRY2 clusters poorly in its wild-type form and thus requiring a high local CRY2 concentration, directed mutagenesis (E490G), or association with multivalent or tagging proteins to generate a more robust clustering effect that can be leveraged as tools to probe and manipulate protein and biomolecular condensates activities (Dine et al., 2018; Kichuk et al., 2021; Lee et al., 2014; Park et al., 2017; Schneider et al., 2021; Tang, 2019; Taslimi et al., 2014), or regulate flow of metabolites in cells (Fig. 5, Table 2) (Zhao et al., 2019).

VVDs are well-known as homodimers but in fact there are other kinds of regulatory proteins in fungus that VVDs do interact with. FUN-LOV (Fig. 3G) is the optogenetic tool that built upon the interaction between VVD and WC-1, a transcription factor that accounts for photo-adaptation regulation (Malzahn et al., 2010; Salinas et al., 2018; Zoltowski and Crane, 2008). The tool was used in a study to control protein expression and yeast flocculation, and it demonstrated an exquisite induction profile (1 300-fold) and a considerably low baseline activity under both white or blue light circumstances (Salinas et al., 2018).

Not only that, VVDs and other natural photoreceptors can even be engineered to alter their intrinsic light-induced responses. For instance, VVDs have been engineered to associate as a heterodimer through electrostatic interaction by engineering the surface of contact with positively and negatively charged residues (pMag and nMag) (Kawano et al., 2015). This engineered photoswitch is now known as “magnets” that has substantially faster kinetics than the other blue light photodimers, and has been exploited to control transcription factor activity (Fig. 3H) (Baumschlager et al., 2017) or in conjunction with other synthetic biology technology such as DNA recombinant (Sheets et al., 2020) and CRISPR (Nihongaki et al., 2015) in a light-dependent manner.

5. Optogenetic approaches in microbial synthetic biology for bioproduction

The application of optogenetics in microbial systems has started to gain more attention recently. There have been a number of endeavours that applies optogenetic tools in microbes, such as to produce desired metabolites, develop novel therapeutics, promote gut longevity, induce apoptosis of pathogens, and control activity of microbe consortia (Chen and Wegner, 2020; Chernov et al., 2017; Hartsough et al., 2020; Liu et al., 2018a; Losi et al., 2018). In this section, we highlight the synthetic biological application of optogenetics in optimizing the bioproduction in microbial cell factories which again, are limited to the two model microbial chassis – *S. cerevisiae* (yeast) and *E. coli*, given that a very limited optogenetic toolset is available for other non-model microbial hosts.

5.1. *Bidirectional light-controlled metabolic valves*

In yeast, Zhao and co-workers have attempted to optically control the transition between two-phase fermentation by manipulating pyruvate distribution across the competing pathways (Zhao et al., 2018). Inspired by the robustness and the bidirectional regulatory circuit design of EL222 scaffold (Jayaraman et al., 2016; Motta-Mena et al., 2014), they have constructed a light-induced growth system, Opto-EXP and a light-suppressed production system, Opto-INVRT that collectively constitute a bidirectional light-controlled “metabolic valves” system (Fig. 4). To initiate the shift towards microbial cell growth, Opto-EXP is programmed to induce transcription of pyruvate decarboxylase

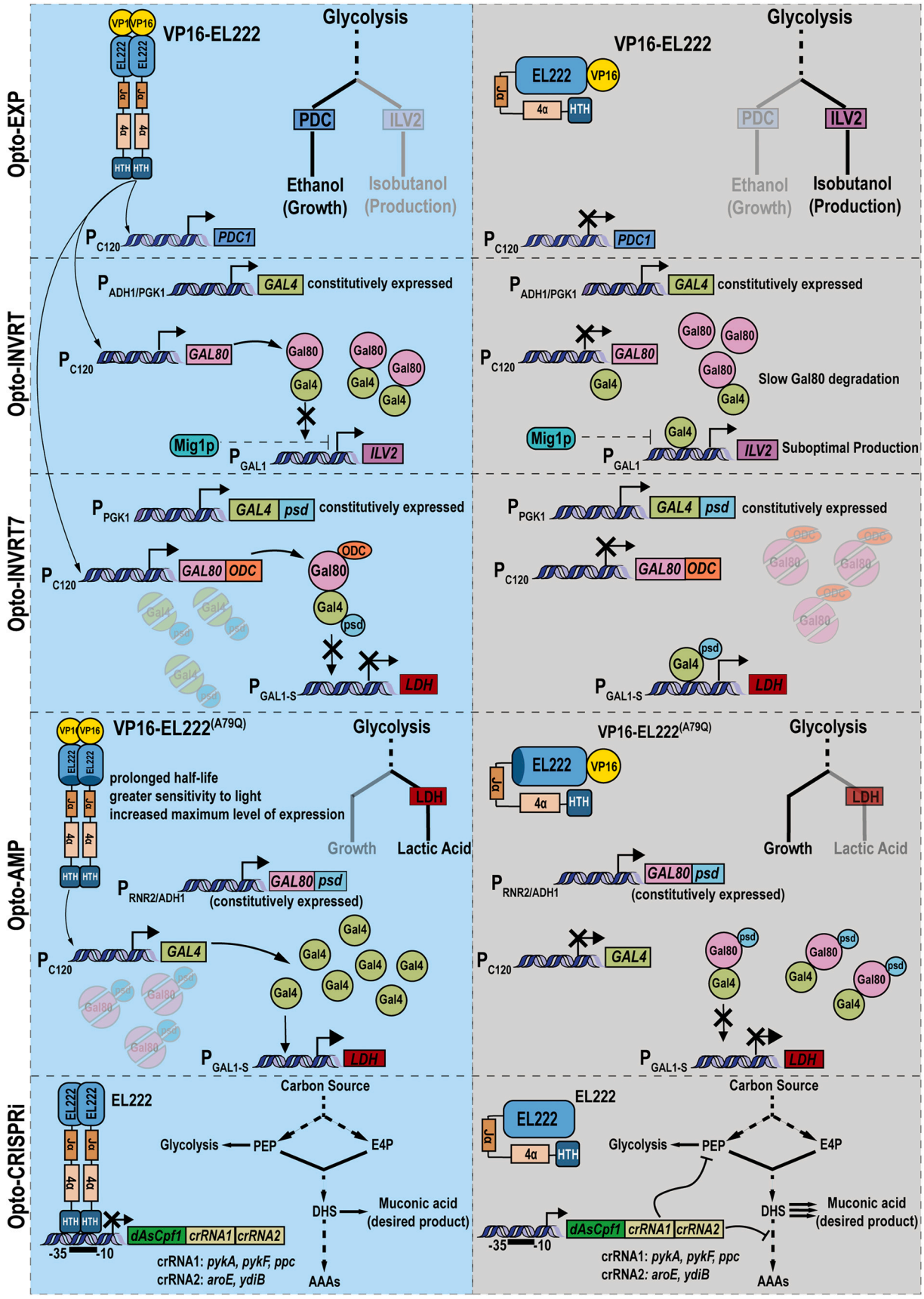

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Fig. 4. Applications of optogenetic systems for optimization of metabolic flux and bioproduction in microbial cell factories are shown above. The coloured boxes represent the light conditions such as blue (λ ≈ 450 nm), red (λ ≈ 660 nm), green (λ ≈ 535 nm) and darkness. Opto-EXP, Opto-INVRT and Opto-INVRT7 systems are developed using the photoreceptor EL222 conjugated with the transcriptional factor VP16 (VP16-EL222). In the Opto-EXP system, VP16-EL222 switches on transcription of *PDC1* gene (encodes for pyruvate decarboxylase 1) from the promoter P_{C120} for cellular growth when treated with blue light. Opto-INVRT which governs the bioproduction pathway is activated under dark condition as the expression of transcriptional repressor Gal80 is inhibited, so that the constitutively expressed transcriptional activator Gal4 protein can bind to DNA to activate the transcription of *ILV2* gene (encodes for acetolactate synthase) from promoter P_{GAL1} for isobutanol synthesis. Opto-INVRT7 system is an optimized version of Opto-INVRT integrated with photosensitive degron (psd) and murine ornithine decarboxylase degradation (ODC) modules to regulate the intracellular level of transcription activator (Gal4) and its repressor Gal80 through protein degradation respectively, as means to improve dynamic range of the system. Opto-AMP system involves a hypersensitized VP16-EL222 through a A79Q mutation to enable the expression of *LDH* gene (encodes for lactate dehydrogenase) and production of lactic acid under limited light source. In Opto-CRISPRi, the dCas12a-mediated CRISPRi system (previously known as dCpf1) that is in charge of repressing the expression of muconic acid production competing pathways genes (as encoded in the *crRNA1* and *crRNA2*) is in turn modulated by the EL222 in a blue light-dependent manner. Abbreviations: AAAs, aromatic amino acids; crRNA, CRISR RNA; dAsCpf1, catalytically deactivated CRISPR-Cpf1 from *Acidaminococcus* sp. BV3L6; DHS, 3-dehydroshikimate; E4P, D-erythrose 4-phosphate; PEP, phosphoenolpyruvate.

(encoded in *PDC1* gene) on promoter P_{C120} upon activation of VP16-EL222 under blue irradiation, promoting pyruvate conversion into ethanol and cell growth on glucose. Concomitantly, the Opto-INVRT circuit governing the bioproduction (either lactate, isobutanol or 2-methyl-1-butanol(2-MBOH)), is repressed under blue light via a NOT gate-alike mechanism, whereby the photoactivated VP16-EL222 activates the expression of the Gal80, which is a repressor of Gal4, to inhibit the transcription of metabolic genes at the downstream of the Gal4-responsive promoter, P_{GAL1} (Fig. 4).

The coalescence among Opto-EXP and Opto-INVRT (Fig. 4) generates a bidirectional system of tighter control. When applying periodic light pulses to the bioreactor during the production phase (dark), NAD^+ is replenished alongside the transiently expressed Pdc1, which therefore can avoid metabolic arrest and improve the production in overall. Under the pulsatile induction protocol, the microbial production of isobutanol and 2-MBOH are superior, producing yields that surpass the maximum recorded titre in literature by 5- and 20-folds correspondingly (Zhao et al., 2018).

Due to the slow nature of protein degradation, the Gal80 repressive protein continues to block Gal4 activity in the dark, thus leading to a delayed response in transitioning to the production phase in the Opto-INVRT system. This diminishes the production profile since Gal4 is essential for the transcription of the metabolic enzymes that account for the production. To circumvent this, the abundance of Gal80 and Gal4 are regulated by a constitutive degradation tag (Gal80-ODC) and a psd module (Gal4-psd), respectively to offset the leakiness in both light and dark phases and increase the overall efficiency of the circuit (Zhao et al., 2020). In addition, Zhao and co-workers enhance the strength of gene expression using a Super GAL1 Promoter ($P_{GAL1\text{-}S}$) which is engineered through the removal of endogenous repressor binding sites (Mig1p) and the introduction of four extra copies of Gal4 upstream activating sequence to the promoter. Finally, the optimized construct, Opto-INVRT7 (Fig. 4), which displays a more robust gene regulation with more than 130-fold induction window and 7-fold faster activation kinetics, has increased the production profiles of isobutanol and lactate by 50% and 15% than that of the original Opto-INVRT respectively (Zhao et al., 2020).

5.2. Amplified optogenetic circuit for photoinduced production

While engineering the microbial chassis to adopt a dark production phase has been proven effective in addressing light penetration issue in high cell density environments, there is a concern, where after shifting the system towards production, the subsequent light induction to revert it to the growth phase might become difficult because of the accumulation of products in the reactor. As such, Zhao and co-workers have taken up another approach to redesign the Opto-EXP system to attain higher light sensitivity and greater transcriptional output to allow the chemical production to proceed under light induction.

Several modifications have been made to create the new series of blue light-activated Opto-AMP circuits (Fig. 4) (Zhao et al., 2021). Firstly, the Gal4 expression is made inducible by VP16-EL222 under promoter P_{C120}, so that the downstream metabolic genes in the Gal4-responsive promoter $P_{GAL1\text{-}S}$ will be transcribed under blue light as well. The VP16-EL222 is engineered through a previously reported A79Q substitution (Rivera-Cancel et al., 2012) to confer a prolonged activation state ($t_{1/2}$ is increased by 10-fold) for a more sustained production stage when there is a limited source of light. However, further amendment is required to counteract the leakiness caused by the prolonged photoactivation of EL222 and restore a tight OFF-state repression whilst maintaining the superior expression level. As such, they introduced the constitutively expressed Gal80 repressor with a psd module (Renicke et al., 2013) so that it can act against leaky Gal4 in the dark but immediately degraded by the psd module upon light illumination, leaving the Gal4 protein with maximal activity under light condition but tightly repressed in the dark.

When simulating the conditions in industrial settings such as low light induction (5s ON/95s OFF), high cell density of up to 41 OD_{600} in a large scale bioreactor (5L), the OptoAMP is able to demonstrate a robust gene expression while OptoEXP is unable to do so (Zhao et al., 2021). Notably, implementing different light pulse schedules throughout the novel three phase fermentation protocol developed by the authors has yielded optimal production of lactic acid, isobutanol, and naringenin. Hence, their findings suggest that OptoAMP can improve chemical production by rationally designing light schedules at different stages of fermentation and imply its future application in more complex metabolic pathways and also in larger-scale bioreactors.

5.3. Light-regulated metabolic channelling

In most cases, despite having the metabolic gene clusters expressed in the host, reaching an optimal and economically viable production titre remains challenging due to inefficiencies in the metabolic channelling of fluxes and utilization rate of substrate along the biosynthesis route (Yang et al., 2020a). These are likely to collectively cause the accumulation of toxic intermediates in hosts and also misallocation of the substrate into competing pathways to produce unwanted by-products, thereby diminishing the optimal production titre. To improve metabolic channelling, which in turn shall boost the efficiency of the desired bioproduction, several modalities have been proposed along with demonstrations of effectiveness in attaining the goal, such as localization of metabolic enzymes to synthetic scaffolds (Dueber et al., 2009), construction of artificial transport metabolon to directly feed in the necessary enzymes spatially (Thomik et al., 2017) and enzyme agglomeration (Castellana et al., 2014).

In this regard, Zhao and co-workers employed the previously described light-induced clustering and phase separation properties of Cry2olig (Taslimi et al., 2014) and PixELL (PixD/PixE) (Dine et al., 2018) systems to mediate the channelling of metabolic substrates within a metabolic pathway (Zhao et al., 2019). The production of deoxyviolacein involves a series of enzymatic reactions and has two distinct endpoints depending on the enzymatic activities of VioE and VioC. VioE catalyses the production of an intermediate, protodeoxyviolaceinate (PTDV) which can either be converted into the desired pink-coloured

deoxyviolacein via VioC, or, a green-coloured prodeoxyviolacein by-product through a non-enzymatic pathway. In the engineered optogenetic clustering systems, VioE and VioC are assembled into synthetic organelles under blue (OptoCluster) or dark (PixELL) circumstances (Fig. 5), in order to selectively drive the enzymatic processes of the intermediate substrate and conversion into the desired deoxyviolacein. This compartmentalization strategy stands out as an optical post-translational regulation of metabolic flux to improve the efficiency of metabolic channelling and chemical production by 6- and 18-fold, respectively, without interfering the gene regulatory machinery (Zhao et al., 2019).

5.4. Light-regulated intracellular cofactor and flux

In microbial chemical production, the availability of intracellular cofactors such as ATP, NAD^+ and NADPH plays a key role in achieving an optimal bioproduction (Toya and Shimizu, 2020; Yang et al., 2020a). In particular, NADPH is highly consumed in the production of various highly-reduced compounds, including mevalonate and isobutanol (Tandar et al., 2019). Therefore, the *in situ* replenishment of NADPH is vital for a sustained and maximized production in microbes as to avoid the accumulation of intermediate substrates and metabolic arrest in the hosts.

In a previous study, the replenishment of NAD^+ during metabolism was partially done using pulses of blue light (Zhao et al., 2018). In *E. coli*, the Embden-Meyerhof-Parnas (EMP) and oxidative pentose phosphate (oxPP) are the two major glycolytic pathways with the oxPP pathway, in particular, being able to regenerate NADPH at the expense of carbon atom (Kamata et al., 2019). In light of this, they are put under the regulation of green and red signals to improve NADPH intracellular abundance and to enhance the productivity in bioprocess (Tandar et al., 2019). The histidine kinase photoreceptor CcaS and its response regulator, CcaR constitutes a TCS that is sensitive to both green and red irradiation. Since the *pgi* gene is the branch point between EMP and oxPP pathways, it is controlled by promoter P_{cpcG2} and CcaS/CcaR so that it is expressed under green light to enter the EMP pathway, but inhibited under red light to redirect the flow of flux into oxPP pathway to regenerate NADPH (Fig. 5) (Tandar et al., 2019). In this work, an engineered CcasR-*pgi* ver.3 *E. coli* strain that consisted of a miniatured *ccaS* gene (Nakajima et al., 2016; Ong and Tabor, 2018), a lowered plasmid copy number, and a ssRA-tagged *pgi* gene, was implemented to minimize the basal leakiness and refine the dynamic range of the EMP flux induction between 0.5% in red light and 50.4% in green light (Tandar et al., 2019). Other attempt using isopropyl β-D-1-thiogalactopyranoside (IPTG) induction has also been conducted, but giving a lower EMP flux induction (39.7%) in comparison with CcaS/CcaR (Kamata et al., 2019).

Following that, Senoo and co-workers further described the use of CcaS/CcaR system in optimizing microbial productivity and alleviating accumulation of toxic intermediates in *E. coli*. To redistribute the metabolic flux between glycolysis and methylglyoxal (MGO) pathways, the *tpiA* gene (encodes for triosephosphate isomerase (TPI)) was targeted to promote metabolism of dihydroxyacetone phosphate (DHAP) through the glycolysis pathway instead of the MGO pathway that will produce the toxic MGO intermediate (Senoo et al., 2019). By placing the *tpiA* gene under the control of promoter P_{cpcG2}, the green light-activated CcaS/CcaR system will express TPI that catalyses the conversion of DHAP into glyceraldehyde 3-phosphate (GAP), thereby directing the glucose metabolism via glycolysis pathway (Fig. 5). Conversely, the *tpiA* gene is repressed under red illumination and therefore DHAP undergoes the alternative MGO pathway to produce lactate. Despite the leaky expression of *tpiA* under red illumination, the model was able to alleviate the accumulation of the toxic MGO by 68% using optogenetic approach (Senoo et al., 2019).

5.5. Optogenetic control of lac operon

Lac operon is a universal bacterial gene expression system derived from *E coli* and inducible by isopropyl β-D-1-thiogalactopyranoside (IPTG). As mentioned early on, the use of such a chemical inducer is often accompanied by pharmacokinetic complications, toxicity and cannot be removed nor added at will to adjust the input and output. Thus, Lawani and co-workers have lately devised an optogenetic circuit, OptoLAC that made up of an engineered *lac* operon which is modulated by blue light instead of IPTG (Lalwani et al., 2021). Theoretically, the circuit shall be compatible for use in any applications that are originally designed based on IPTG and *lac* operon (Lalwani et al., 2021).

OptoLAC is essentially constructed using the previously developed pDawn system (Moglich et al., 2009a; Ohlendorf et al., 2012), with several modifications. In the case of OptoLAC (Fig. 5), a LacI repressor is placed under the control of pDawn system, that in turn, regulates *lac operator (*LacO*)* sites-containing promoters and its downstream genes (Lalwani et al., 2021). With such architecture, as LaCI is expressed in response to blue light, it then represses the GOI as well as the cI repressor, to constitute a positive feedback loop to reduce leakiness in the circuit (Fig. 5). In this work, the portability of OptoLAC was validated in five different LacO-containing promoters, namely $P_{T5\text{-}lacO}$, P_{lacUV5}, P_{trc}, $P_{L\ lacO1}$ and P_{T7}. Furthermore, the system was adopted as a metabolic valve to temper the transition in between the two-phase fermentation, achieving mevalonate and isobutanol production which were higher than IPTG induction by 24% and 27%, respectively (Lalwani et al., 2021).

5.6. Multiplexed optogenetic control of metabolic pathways

CRISPRi is a powerful technique that employs dCas9 and programmable target sequence containing sgRNA to interfere the expression of GOI (Tong et al., 2020). CRISPRi has been employed in synthetic biology and in various microbial chassis to repress competing pathways, but the quest for a dynamic induction of CRISPRi activity to maintain the balance of the metabolic flux distribution for optimal productivity remains untapped due to the chemical inducers such as arabinose and tetracyclines are predominantly used in the systems (Gordon et al., 2016; Tian et al., 2019). Earlier in this review, a multiplexed CRISPRi/dCas9 system (RGB system) which is regulated by three different photoreceptors have been described (Section 4.2.1) (Fernandez-Rodriguez et al., 2017).

In this work, Wu and co-workers have opted for a type V-A CRISPR system, which is based on a Cas12a effector nuclease (previously known as Cpf1) and its cognate CRISPR RNA (crRNA) which are of a more compact toolset and offer greater genetic stability than Cas9 (Wu et al., 2021; Zetsche et al., 2015). Using the catalytically deactivated Cas12a mutant (dCas12a) and EL222, genes in the competing pathways of the muconic acid biosynthesis are manipulated under blue light cultivation in order to improve microbial cell productivity (Fig. 4). Particularly, the authors have constructed two crRNA arrays (*ppc-pykF-pykA*) and (*aroE-ydiB*) containing the competing enzyme genes that are combined and placed under promoter P_{T7} to be induced under dark cultivation (Wu et al., 2021). This optogenetic system named Opto-CRISPRi offers dynamic regulation of CRISPRi activity to repress metabolic pathways and has significantly raised muconic acid production by up to 130% in the study (Wu et al., 2021).

5.7. Optochemical-controlled metabolic switch

Raghavan and co-workers have extended their previous work on the temperature-responsive T7 RNA polymerase (T7 RNAP) (Korvin and Yada, 2018) by reengineering it into a temperature and red light-responsive entity. Switching biomass accumulation and production of lycopene in *E. coli* at a specific temperature (18°C) has shown effectiveness in precluding metabolic stress and attaining more superior lycopene production. Nevertheless, this approach is likely to be impeded

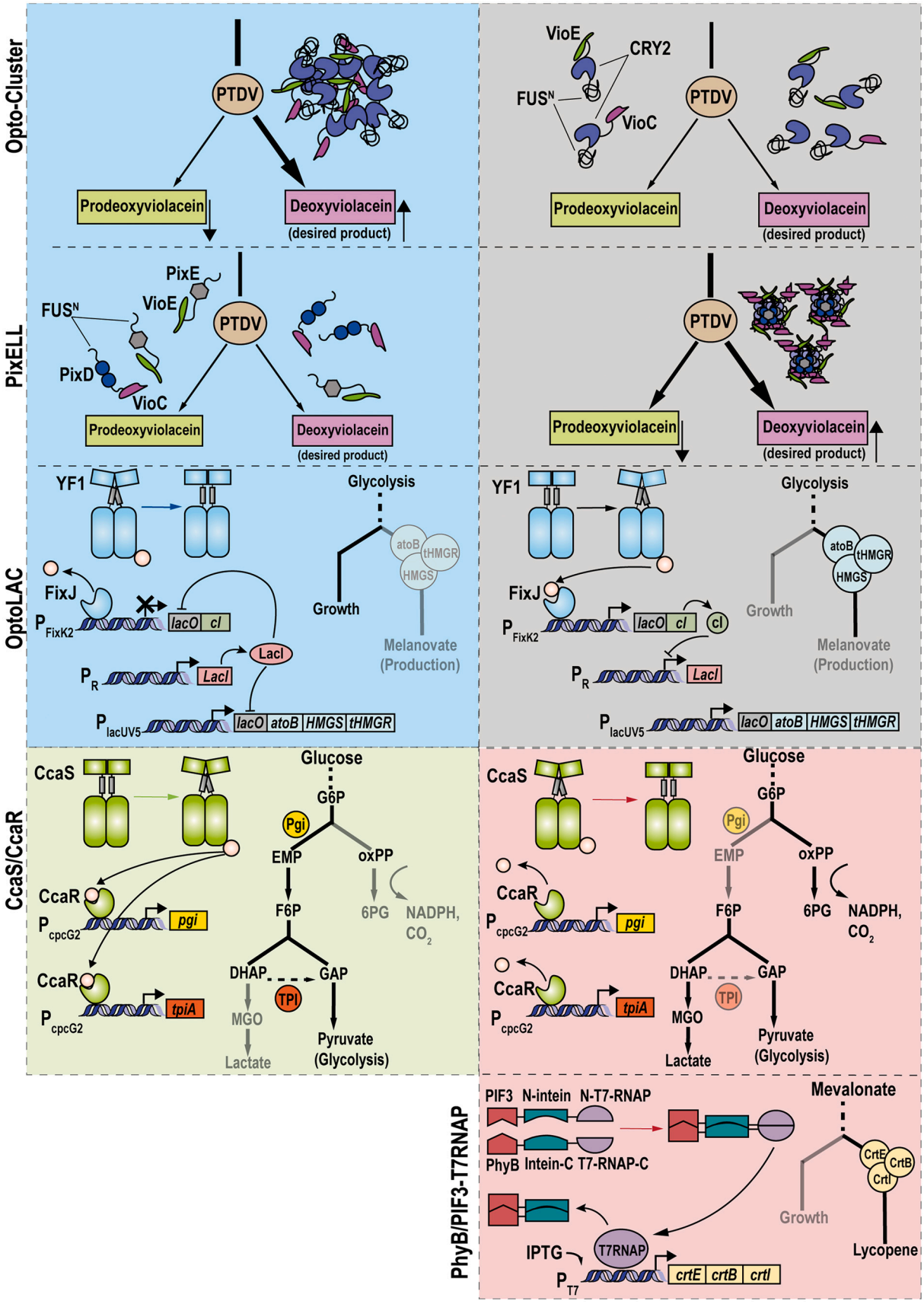

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Fig. 5. Applications of optogenetic systems for optimization of metabolic flux and bioproduction in microbial cell factories are as shown above (continued). The coloured boxes represent the light conditions such as blue (λ ≈ 450 nm), red (λ ≈ 660 nm), green (λ ≈ 535 nm) and darkness. Opto-Cluster and PixELLs systems are adopted and directly fused with metabolic enzymes to form synthetic organelles scaffolds and improve metabolic channelling under different light conditions. OptoLAC is an engineered blue-light responsive *lac operon* in *E. coli* that designed based on the previously constructed pDawn system and further modified to control the switching between growth and production phase. The two CcaS/CcaR optogenetic systems (Senoo et al., 2019; Tandar et al., 2019) are drawn together as shown in the figure above. The CcaS/CcaR two-component optogenetic system that controls the expression of *pgi* gene regulates the abundance of nicotinamide adenine dinucleotide phosphate (NADPH) cofactor which is essential for enzymatic reactions. In another case, the promoter P_{cpcG2} which encodes for the *tpiA* gene is used to drive glycolysis when treated with green light and bypass the methylglyoxal (MGO) pathway that produces toxic intermediate. PhyB/PIF3 regulated T7RNAP system is irreversible. When treated with red light, PhyB and PIF3 heterodimerize to reconstitute the functional VMA inteins, which then cleaves off the reconstituted T7RNAP to switch on the transcription of metabolic genes for lycopene synthesis. Abbreviations: 6PG, 6-phosphogluconate; *atoB*, acetoacetyl-CoA thiolase; DHAP, dihydroxyacetone phosphate; F6P, fructose 6-phosphate; FUS^N, N-terminal intrinsically disordered region (IDR) of RNA binding protein FUS; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GOI, gene of interest; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; *lac* repressor protein; tHMGR, truncated 3-hydroxy-3-methylglutaryl-CoA reductase; *lacO*, *lac* operator; cI, λ repressor; LacI, *lac* repressor; Pgi, phosphoglucose isomerase; PTDV, protodeoxyviolaceinate; TPI, triosephosphate isomerase; VioC, proviolaceinate monooxygenase; VioE, violacein biosynthesis enzyme.

for large-scale industrial use because to maintain and adjust to a steady temperature throughout a large bioreactor is challenging since the rate of heat transmission always varies.

Raghavan and co-workers (Raghavan et al., 2020) have therefore reengineered this circuit and rendered it to be optical controlled using plant phytochromes – PhyB/PIF3 and adopting designs involving VMA inteins and the magnets system in Opto-T7RNAPs, that were established in previous studies (Baumschlager et al., 2017; Shimizu-Sato et al., 2002; Tyszkiewicz and Muir, 2008). Interestingly, the operation of the circuit for lycopene synthesis in *E. coli* that they designed requires both chemical and light induction. In this design, the red light-inducible dimerization property of PhyB/PIF3 is leveraged to control the reorganization of split VMA intein and T7 RNAP (Fig. 5). On the other hand, the expression of the crucial PCB cofactor biosynthesis pathway, as well as the genes which account for the lycopene production are assigned under promoter P_{T7} that proceeds via the induction of IPTG. The incubation of IPTG triggers the synthesis of PCB required by the phytochrome, but the lycopene production cannot proceed until T7 RNAP binds to the genes. When exposed to red light, the dimerization of PhyB/PIF3 restores the function of VMA intein to splice and liberate a functional T7 RNAP to initiate transcription on promoter P_{T7}. Notably, this series of operations irreversibly uncouple bioproduction from cell growth as the VMA intein trans-splicing activity cannot be undone. Therefore, an appropriate timing to illuminate red light is very crucial in which the transition should be made when an appropriate biomass level has been reached to achieve maximal production and prevent invocation of metabolic burden on the hosts.

5.8. Optogenetic control of biofilm-based biocatalyst

In nature, microorganisms live in social communities, presenting themselves as surface-attached consortia to cooperate and communicate with one another, synergistically carry out complex tasks and become more resilient to harsh environments to increase their chance of survival (Flemming and Wingender, 2010; Zhang et al., 2020). Such microbial consortia are commonly known as biofilms. The spatial organization of biofilm communities makes them a double-edged sword, in which the biofilm matrix can serve as a protective barrier against host's immune responses and antimicrobials (Costerton et al., 1999), or it can be viewed as potential biomaterials for biotechnological or biomedical applications by manipulating their formation and structure spatially and temporally (Bjarnsholt et al., 2018; Chen and Wegner, 2017; McCarty and Ledesma-Amaro, 2019; Muhammad et al., 2020; Sonawane et al., 2022). To exploit their potential, advanced and efficient tools are much needed and for that, light approach is appealing in this context because it can be delivered remotely in patterns through an unprecedented spatiotemporal resolution.

Optogenetics has been used to dynamically control the bacteria motility and biofilm formation with patterns (Chen and Wegner, 2017, 2020; Huang et al., 2018; Jin and Riedel-Kruse, 2018; Pu et al., 2018; Zhang et al., 2020). With these tools in hand, the dynamics of biofilms can be engineered to perform various tasks such as biocatalysis. The formation of biofilm can be mediated through c-di-GMP signalling cascade, whereby the biofilm formation increases with the c-di-GMP level in cells (Bjarnsholt et al., 2018; Liang, 2015). To harness the structural feature of biofilm to provide natural immobilization and physical protection against physiochemical stress, Hu and co-workers have made the first demonstration of using light-regulated biofilm to enhance chemical synthesis using the previous construct, BphS (Hu et al., 2019; Ryu and Gomelsky, 2014). *E. coli* biofilm was engineered to be regulated by NIR light via BphS and capable of catalysing biotransformation of indole to tryptophan by integrating a tryptophan synthase (TrpBA) from *Salmonella enterica* (Hu et al., 2019). To achieve optimal catalytic activity and avoid high mass-transfer limitation, the biofilm was adjusted to a predetermined optimal thickness via NIR light, and ultimately produced tryptophan that was 30% more than that of under dark circumstances (Hu et al., 2019). However, the limitation of this strategy is the lack of a bidirectional regulation to alter the biofilm thickness dynamically, thus further work is much needed.

6. Conclusions and future perspectives

As discussed above, the diversity and modularity of natural photoreceptors have rendered different kinds of biological processes at various levels to be accessible for precise and dynamic regulation by light. However, the current practice of optogenetics in microorganisms is rather confined to small-scale studies and with many are still focusing only on the two most commonly used model chassis: *E. coli* and yeast, which represent a very small portion of the microbial diversity in nature. In fact, many other microorganisms have acquired unique phenotypes and characteristics to adapt to and thrive in extreme environments through evolution, which in turn, can be leveraged to potentially generate novel and sustainable solutions against real-world issues covering the aspects of food, health, energy, and environment using synthetic biology approaches. With this notion in mind, it is then very important to possess precise and efficient tools for exploitation. Even though non-model microorganisms such as streptomyces are well-known as treasure trove of natural products and they have received immense attention to be explored as cell factories, their adoption is largely impeded due to the lack of comprehensive information about their physiology and also synthetic biology tools for intervention (Choi et al., 2019; Palazzotto et al., 2019; Riley and Guss, 2021; Tong et al., 2019; Weber et al., 2015). Optogenetics offers great promise in this premise due to its tunable control to interrogate the complexity of biological network *in vivo* and also to manipulate their biological functions for synthetic biology applications.

Nevertheless, the broad usability of this technology is limited by several factors. First, the heterologous expression of photoreceptor proteins in different hosts can be very difficult that requires further genetic engineering effort to resolve. Additionally, it could also be the photoreceptor proteins that are not compatible with the biological environment in the target host as they may tend to operate differently

across different biological contexts (Tschirhart et al., 2019). As the result, this requires photoreceptor engineering or adjustment on the optogenetic circuit to ensure the feasibility and effectiveness of the control, but at the same time, this demands an extensive knowledge regarding the complex molecular dynamics that are involved during the photoreceptor activation and signal propagation to the effector. Despite the many successful applications of optogenetic systems, the exact mechanisms underlying the structural changes within the photoreceptor to induce to designated biological functions remain poorly understood and the atomic resolution structures for some of them have not been elucidated either (Banerjee and Mitra, 2020; Lu et al., 2020; Möglich and Moffat, 2010; Ziegler and Moglich, 2015). Studies in this regard is still underway and with more of these structural and mechanical insights made available using computational tools in the future (David et al., 2022), they shall provide better guidance and facilitate the rational design to produce variants with superior activities in different hosts. With that being said, in the near future, it will also be very beneficial if one could come up with a set of universal optogenetic systems that correspond to different colours of light, and offer orthogonality as well as compatibility in a broad range of heterologous hosts at the same time. In this way, they can be put into practical use directly without additional considerations and designs.

Besides that, whether optogenetics can be reliably used in large-scale industrial application is concerned by two factors, which is the light penetration efficiency and tolerance of microbes to prolonged irradiation. All colours of light have the propensity to induce phototoxicity and overheating in cells if exposed to light source for too long, but especially for light of shorter and more energetic wavelength such as blue light, the effect is more detrimental to the cell viability. Moreover, light of shorter wavelength has limited penetration depth and thus will result in cell heterogeneity in the fermentation culture. Although one can invert the circuit to make gene expression proceeds in the dark to bypass the light penetration issue (Lalwani et al., 2021; Zhao et al., 2020), the problem still exists. The use of light duty cycle can potentially address this complication, as exemplified by Zhao and co-workers who demonstrated the utilization of about 1% light duty cycle along with the engineered hypersensitive OptoAMP to enhance production of desired products (Zhao et al., 2021). This strategy is appealing from an economic point of view as it requires less light energy for the operation and therefore it is more energy- and cost-saving. However, this strategy requires photoreceptors that are more sensitive to light and have fast kinetics to respond to the short pulse of light, and the minimal light dosage needed for successful induction as well as the appropriate illumination intermediate need to be worked out as this would vary across different application settings. Photobioreactor design and internal illumination present another potential solution but they might require the modelling of light distribution to help with the optimal designs (Pouzet et al., 2020). Alternative approaches could be developing appropriate temperature control system in the bioreactor to compensate the heat produced by prolonged light exposure, adaptive evolution to improve the tolerance of microbial hosts to the light exposure, or formulate specific culture media that can help to counteract the phototoxicity (Stockley et al., 2017).

Other than that, to push forward optogenetics to the system-level applications which are of more biological relevance, a light-regulated multiplexed platform that reacts with varying light wavelengths is required for dynamic regulation of the multidimensional biological network. Nevertheless, this is not an easy task because of the complex circuit architecture involved, where multiple sets of optogenetic systems need to be integrated into the same cell and make sure they function coherently while at the same time avoiding the cross-interaction between the photoreceptors of overlapping wavelengths and unwanted side effects (Dwijayanti et al., 2021). In addition to that, the heterologous expression of these bulky systems, along with BV or PCB cofactors (in the case of phytochromes or CBCR is used) may not be feasible in the target host due to limited capacity or metabolic burden (Dwijayanti et al., 2021). This calls for establishment of more compact systems (Ohlendorf et al., 2012; Schmidl et al., 2014) and miniatured photoreceptors (Nakajima et al., 2016; Ong and Tabor, 2018) to alleviate this concern.

At present, we are in the state where we are harnessing the natural scaffold and functions of photoreceptors with minimal engineering such as random or site-directed mutagenesis to optimize or alter its performance (Kawano et al., 2015; Taslimi et al., 2016; Zoltowski et al., 2009). With the advent of modern biotechnology such as enzyme directed evolution (Chen and Arnold, 1991; Herwig et al., 2017; Yang et al., 2019), and de novo protein design (Huang et al., 2016; Kuhlman et al., 2003), together with the aid of artificial intelligence and advanced algorithms for protein structure prediction (Senior et al., 2020; Yang et al., 2020b), it is highly envisioned that we can push forward optogenetics to a more synthetic and diversified level by engineering existing photoreceptors into variants of distinct wavelength specificity, (like how variants Green Fluorescent Proteins were developed (Stepanenko et al., 2008)), or entirely new photoreceptors with novel modes of action. These will in turn, enrich the assortment of optogenetic toolkits and promote the development of microbial synthetic biology and for a more sustainable world.

Author credits

Sang Yup Lee and **Yaojun Tong:** Conceptualization, Funding acquisition; **Natalie Chia**, **Sang Yup Lee** and **Yaojun Tong:** Writing - Original draft preparation, Writing - Reviewing and Editing and Visualization.

Declaration of Competing Interest

The authors of this manuscript (Natalie Chia, Sang Yup Lee, and Yaojun Tong) declare no competing financial interests.

Data availability

No data was used for the research described in the article.

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