Review

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Tansley insight

Optogenetics in plants

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Summary

The last two decades have witnessed the emergence of optogenetics; a field that has given researchers the ability to use light to control biological processes at high spatiotemporal and quantitative resolutions, in a reversible manner with minimal side-effects. Optogenetics has revolutionized the neurosciences, increased our understanding of cellular signalling and metabolic networks and resulted in variety of applications in biotechnology and biomedicine. However, implementing optogenetics in plants has been less straightforward, given their dependency on light for their life cycle. Here, we highlight some of the widely used technologies in microorganisms and animal systems derived from plant photoreceptor proteins and discuss strategies recently implemented to overcome the challenges for using optogenetics in plants.

I. Introduction

Optogenetics refers to the use of light-responsive proteins to control and dissect biological functions. Light-responsive proteins are abundant in nature and have been instrumental in generating an ever-growing suite of tools for optogenetic applications (Goglia & Toettcher, 2019). As these tools are genetically encoded, they can be introduced into any cell type of choice and used, together with optical methods, to artificially manipulate a specific signalling output. Light provides a powerful control tool as it can be delivered within defined spaces, and can elicit rapid, reversible and quantitative effects following photoreceptor activation with minimized toxicity.

The incentive for optogenetics came from the need to develop a technology that could dissect the complexity of the neural circuitry in animals (Deisseroth & Hegemann, 2017). Microbial opsins have contributed considerably in this regard, with these light-activated ion pumps and ion channels being used as first-generation optogenetic tools (Box 1) to introduce electrochemical changes across cellular membranes that can, in turn, excite or inhibit neural activity depending on their ion transport properties. Optogenetics now extends beyond the regulation of membrane potential changes. A second generation of optogenetic tools (Box 2) has been successful in placing a wide range of biological processes under optical control (Kolar & Weber, 2017; Krueger *et al.*, 2019). Many of these tools originate from plant photoreceptor proteins (Ziegler

Box 1 Optogenetics 1.0

Engineering of opsin-based light-gated ion channels into animal cells has revolutionized the neurosciences (Deisseroth & Hegemann, 2017). Optogenetics 1.0 began with the seminal work of Nagel, Bamberg, Hegemann, Boyden and Deisseroth, among others, in the early 2000s, which successfully used algal retinal-binding channelrhodopsins (ChRs) to control membrane polarization and thus neuronal activity at high spatiotemporal resolution and reversibility. The following 15 yr witnessed an explosion of optogenetic tools and application, fuelled by a wide range of available ChRs and other opsins, including light-activated ion pumps (halorhodopsins, bacteriorhodopsins). The properties of -opsins have been engineered extensively, aided by structure-function analysis, to generate variants with faster or slower activation/deactivation kinetics, light adaptation/bistability, photosensitivity, colour tuning (wavelength dependency), ion selectivity (H^+, K^+, Na^+, Ca^{2+}, Cl^-) and direction of ion transport (inwards vs outwards). Optogenetics 1.0 has led to major advances in our understanding of neuronal activity dynamics regulating cognition, perception and behaviour in health and disease that is likely to facilitate development of novel intervention therapies. Key advances in associated illumination technologies (optrodes) have also added to the various disciplines contributing to Optogenetics 1.0 (biophysics, biochemistry, neurobiology, biomedicine, material sciences and engineering). Engineering retinal cofactor biosynthesis into plant cells will enable broad application of opsin-based tools, opening up novel perspectives for plant developmental and physiological studies.

Box 2 Optogenetics 2.0

Optogenetics 2.0 deals with the engineering and implementation of photoreceptors for the control of cellular processes with light, going beyond ion transport control, and is being applied to a diversity of organisms ranging from bacteria and fungi to animals and plants (Kolar & Weber, 2017). For this purpose, light-sensitive proteins or domains are engineered to form synthetic photoswitches. They generally consist of two modules: a photosensory module coupled to an effector module of desired function. Key examples are given in the main text. To date, a multitude of photoreceptor sources from bacteria, fungi and plants have been incorporated into optogenetic devices. They vary in photobiology (wavelength dependency, kinetics of activation/deactivation, photosensitivity) and molecular mechanism of function. Their introduction into living cells has led to a myriad of applications, including optical control of gene expression, protein stability, subcellular localization of proteins and organelles, enzyme activity, kinase and receptor activity, and biohybrid materials. Multiplexing photoswitches permits us to achieve independent, orthogonal control over two or more processes simultaneously. Optogenetics 2.0 is still progressing at a fast pace, and current bottlenecks are being overcome. For example, if required, cells can be engineered to express the necessary enzymes required for chromophore production. Despite the advances made, a generalized application of advanced engineering strategies integrating mathematical models are required to optimize and customize photoswitches for their dynamic properties, functionalities and cell/organism of interest (Muller *et al*., 2014a; Ruess *et al*., 2015; Benzinger & Khammash, 2018; Golonka *et al*., 2019). The web-based platform optobase (www.optobase.org) (Kolar *et al*., 2018) provides a valuable resource in cataloguing the diversity of the optogenetic tools available, their different applications and organism of choice.

& Moglich, 2015; Glantz *et al*., 2016). In this review, we highlight both the contributions made by plant photoreceptors to the optogenetic toolkit and how optogenetics is being used to study and manipulate cellular and physiological processes in plants.

II. Plant photoreceptor contributions to the optogenetic toolkit

Plants are a plentiful source of photoreceptors as light is pivotal for regulating their growth and development. PHYTOCHROMES (PHYA-E) absorb mainly in the red/far-red region (Legris *et al*., 2019), whereas cryptochromes (CRY1, CRY2), PHOTOTROPINS (PHOT1, PHOT2) and members of the ZEITLUPE family (ZTL, FKF1, LKP2) respond to ultraviolet (UV)/blue wavelengths (Christie *et al*., 2015). Plants also contain UV-RESISTANCE LOCUS 8 (UVR8) which specifically absorbs in the UV-B region (Christie *et al*., 2012). Together, they absorb across much of the UV/visible spectrum (Fig. 1) and their photosensory properties exploited, singly or in combination, for a variety of optogenetic applications (Banerjee & Mitra, 2020).

Phytochromes bind a linear tetrapyrole chromophore (phytochromobilin), synthesized from heme, that enables them to photoconvert between red and far-red absorbing forms known as Pr and Pfr, respectively (Rockwell & Lagarias, 2010). These properties make PHY particularly attractive for optogenetic applications as independent wavelengths can be used to toggle between active (Pfr) and inactive (Pr) states. Many photoreversible dimerization tools have been created based on PHYs' light-regulated interaction with basic helix–loop–helix (bHLH) proteins known as phytochrome-interacting factors (PIFs) (Fig. 2a). For example, Shimizu-Sato *et al*. (2002) were the first to develop a red light-inducible gene expression system in yeast by fusing protein regions responsible for PHYB–PIF3 interactions to the DNA-binding domain and transactivation domain of GAL4. The utility of PHY-based tools in nonplant systems is limited by the need to supply the chromophore or to synthesize it *in vivo* through genetic manipulation. However, these have been engineered extensively for a variety of applications, including red/far-red control of gene expression, subcellular protein and organelle localization and signalling in mammalian cells (Levskaya *et al*., 2009; Muller *et al*., 2013a) and zebrafish (Beyer *et al*., 2015; Buckley *et al*., 2016) (Box 2).

Interaction modules derived from CRY2 and the transcription factor CRY2 INTERACTING bHLH 1 (CIB1) have also been used as optogenetic dimerizers (Fig. 2b) in mammalian cells to place protein translocation, transcription and Cre recombinase-mediated DNA recombination under blue light control (Kennedy *et al*., 2010). CRYs bind flavin adenine nucleotide (FAD) as chromophore and dimerize following their photoactivation to initiate signalling (Wang *et al*., 2016). The ability to also form homo-oligomers has been exploited to optically modulate protein function through clustering of cellular receptors (Bugaj *et al*., 2015) or steric interference (Fig. 2c).

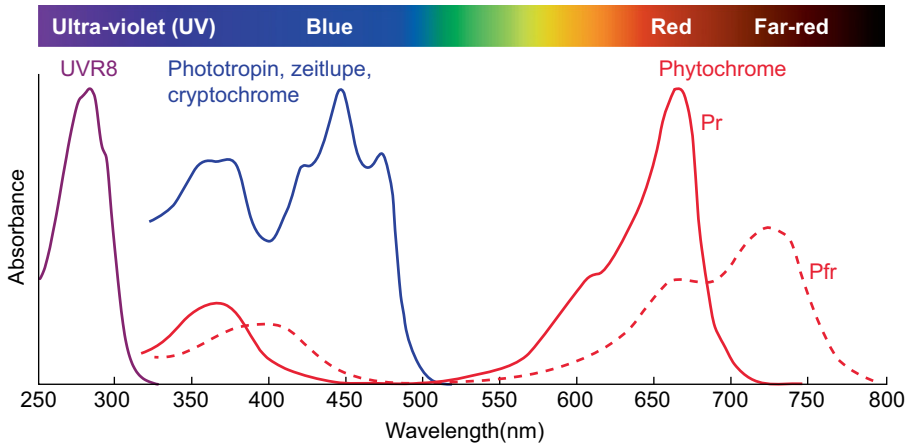

Fig. 1 Plant photoreceptors and their wavelength dependency.

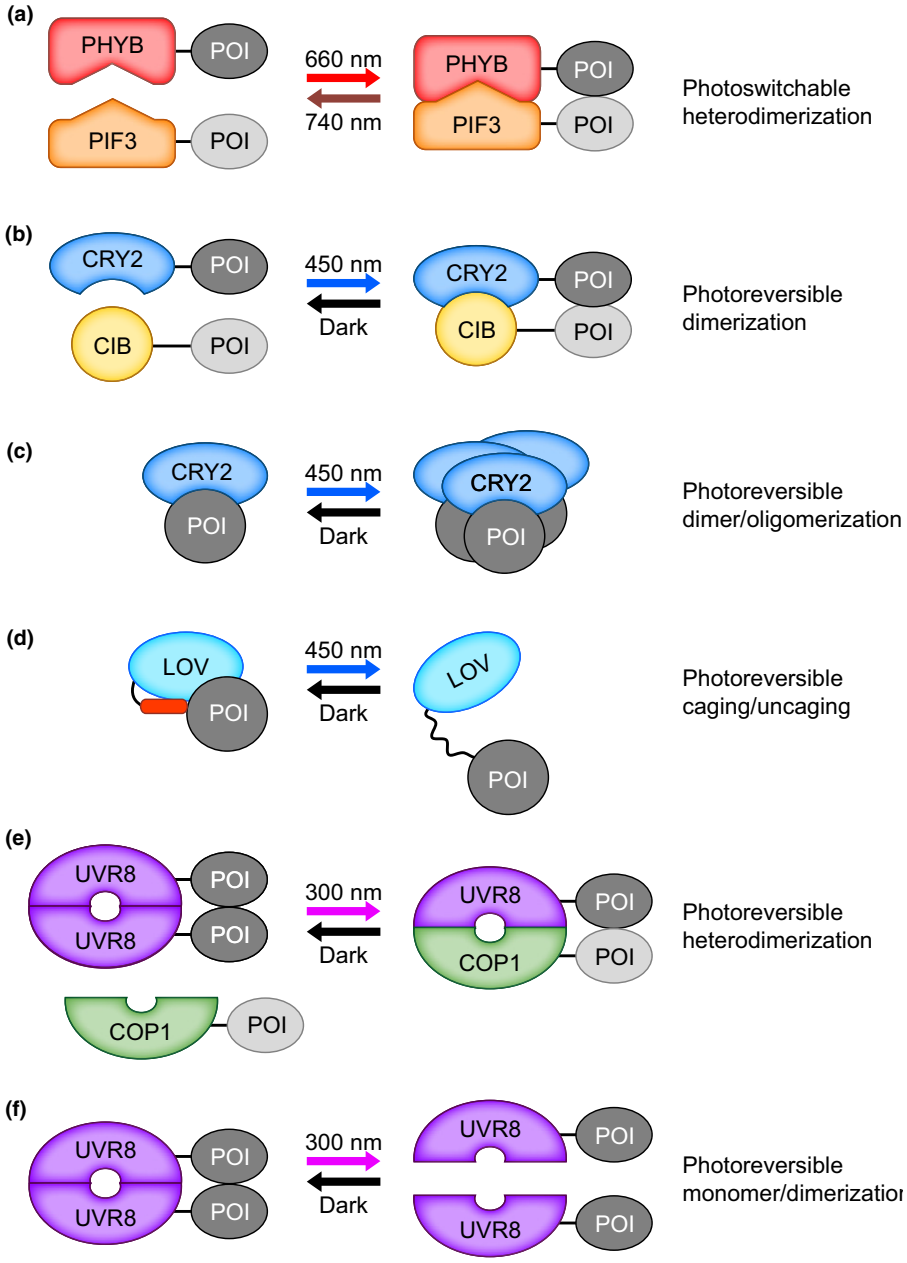

Fig. 2 Optogenetic tool examples derived from plant photoreceptors that are widely used in microbial and mammalian cells. Their mode of action in controlling a protein of interest (POI) is illustrated. (a) Photoswitchable PHYB/PIF heterodimerization. (b) Photoreversible CRY2/CIB heterodimerization. (c) Photoreversible CRY2 dimerization/oligomerization. (d) Photoreversible LOV caging/uncaging. (e) Photoreversible UVR8/COP1 heterodimerization. (f) Photoreversible UVR8 monomerization/dimerization.

For instance, light-induced disruption of clathrin-mediated endocytosis could be achieved when the FAD-binding module of CRY2 was fused to the clathrin light chain (Taslimi *et al.*, 2016). Conversely, CRY2 dimerization has been used to optically activate Raf1 kinase activity (Wend *et al.*, 2014). This process can be fine-tuned to increase or decrease Raf1 signalling by altering the clustering capacity of CRY2 by site-directed mutagenesis (Duan *et al.*, 2017).

Ultraviolet/blue light sensing by PHOTs and the ZTL family is mediated by a photosensory module known as the LOV domain which binds flavin mononucleotide (FMN) as chromophore (Christie *et al.*, 2015). LOV domains regulate protein activities by changing conformation upon irradiation. The photo-uncaging property of the LOV2-Jα photosensory module from oat PHOT1 (Fig. 2d) has been used to develop a range of optogenetic tools (Losi *et al.*, 2018). Fusion of LOV2-Jα to the small GTPase Rac1 represses its activity in darkness through steric interference (Wu *et al.*, 2009). Consequently, actin cytoskeletal dynamics can be controlled remotely upon Rac1 activation by blue light. Optogenetic regulation of Rac1 activity has also been achieved by controlling its plasma membrane recruitment using optogenetic dimerizers based on FKF1's light-regulated interaction with GIGANTEA (Yazawa *et al.*, 2009).

UVR8 is the most recently identified plant photoreceptor and is a homodimer that monomerizes upon UVB exposure to interact with its downstream signalling component CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Rizzini *et al.*, 2011). UVR8 requires no additional chromophore to sense UV-B and does this by a mechanism that involves tryptophan-mediated disruption of cross-dimer salt bridges (Christie *et al.*, 2012). A number of optogenetic applications have been created by hijacking UVR8–COP1 interactions (Fig. 2e), including UV-B-induced gene expression, which, when combined with red and blue light-regulated systems, can afford multichromatic control of transcription in mammalian cells (Muller *et al.*, 2013b). UVR8 has also been used to sequester protein targets at the endoplasmic reticulum and promote their trafficking through the secretory pathway to the plasma membrane following UV-B-dependent monomerization (Chen *et al.*, 2013) (Fig. 2f).

III. Optogenetic control of gene expression in plant cells

Plant optogenetics faces experimental constraints imposed by the fact that light is essential for shaping plant growth and development. This presents a formidable challenge as a plant's dependency on light would inadvertently activate optogenetic tools (Fig. 3a). That said, optogenetics has been applied successfully in plant cells and whole plants. The first demonstration employed a split transcription factor system to control gene expression in *Nicotiana benthamiana* and *Arabidopsis thaliana* protoplasts derived from PHYB–PIF interactions (Fig. 2a). Here, the N-terminal region of PHYB was fused to the VP16 transactivation domain from *Herpes simplex virus* in addition to a nuclear localization signal, whereas the N-terminus of PIF6 was fused to the E DNA-binding domain (Muller *et al.*, 2014b). Red light was used to induce the expression of a luciferase reporter, a microRNA or a phytohormone receptor in transformed protoplasts following reconstitution of the split transcription factor complex and binding to a cognate minimal promoter sequence. This system exhibited a high dynamic range over dark-treated controls (*c.* 400-fold) that could be fine-tuned by varying the intensity of red light. Far-red light terminates PHYB–V16–E-PIF6 interactions, thereby switching the system to an off state. Moreover, supplemental far red repressed reporter activity in the presence of white light and offers a means to circumvent unwanted activation of the system under standard growth conditions (Muller *et al.*, 2014b; Ochoa-Fernandez *et al.*, 2016) with the caveat that endogenous PHY activity might also be affected.

A different engineering strategy was recently developed to help overcome this limitation. A system known as plant usable light-switch elements (PULSE) is insensitive to broad-spectrum white light but activated specifically by red light (Ochoa-Fernandez *et al.*, 2020). It combines two different photoswitches to control transcriptional initiation from a synthetic bipartite promoter: one engineered from the LOV-based transcription factor, EL222 (Nash *et al.*, 2011) (blue-off module), and the other based on the PHYB-PIF6 photoswitch (red-on module) discussed earlier. EL222 dimerizes and binds to its target DNA sequence in blue light (Nash *et al.*, 2011) and can inhibit transcriptional activation when fused to the EAR repressor domain designated SRDX (Ochoa-Fernandez *et al.*, 2020). Thus, PULSE enables effective optogenetic manipulation of plant gene expression under standard growth conditions as transcription is repressed in white light by the blue-off module and is activated only in monochromatic red light by the red-on module (Fig. 3b,c). PULSE has already proved successful in whole plants, as well as protoplasts, and has shown promising potential for a variety of applications, including light-dependent control of plant immunity.

Green/yellow light (500–600 nm) is an attractive wavelength for optogenetic control in plants as native photoreceptors absorb minimally within this region (Fig. 1). A green light-responsive system was recently engineered from the bacterial photoreceptor CarH to control gene expression. CarH uses a vitamin B12 derivative, adenosylcobalamin (AdoB12), as chromophore to regulate its function as transcription factor. CarH binds to the DNA operator CarO as a tetramer in darkness, leading to repression of the carotenogenic target genes, whereas green triggers a disassembly of the CarH tetramer to initiate gene expression. The utility of the CarH photoswitch has already proven useful for controlling reporter gene expression in Arabidopsis protoplasts (Chatelle *et al.*, 2018). However, a disadvantage of the CarH system for *in vivo* applications is that it relies on addition of the AdoB12 chromophore, as this is not produced in plants. It is also becoming more apparent that green wavelengths are effective in stimulating photoreceptor function in plants (Battle & Jones, 2020).

IV. Optogenetic strategies to manipulate K^+ transport and plant physiology

Optogenetic tools are now being used to augment plant responses to light with the same wavelength dependency. Fusion of the LOV2-Jα photoswitch to the miniature K^+ channel Kcv from the Chlorella virus PBCV-1 resulted in a synthetic, blue light-induced K^+ channel, designated BLINK1 (Cosentino *et al.*, 2015). BLINK1 is essentially closed in darkness and opens in response to blue light

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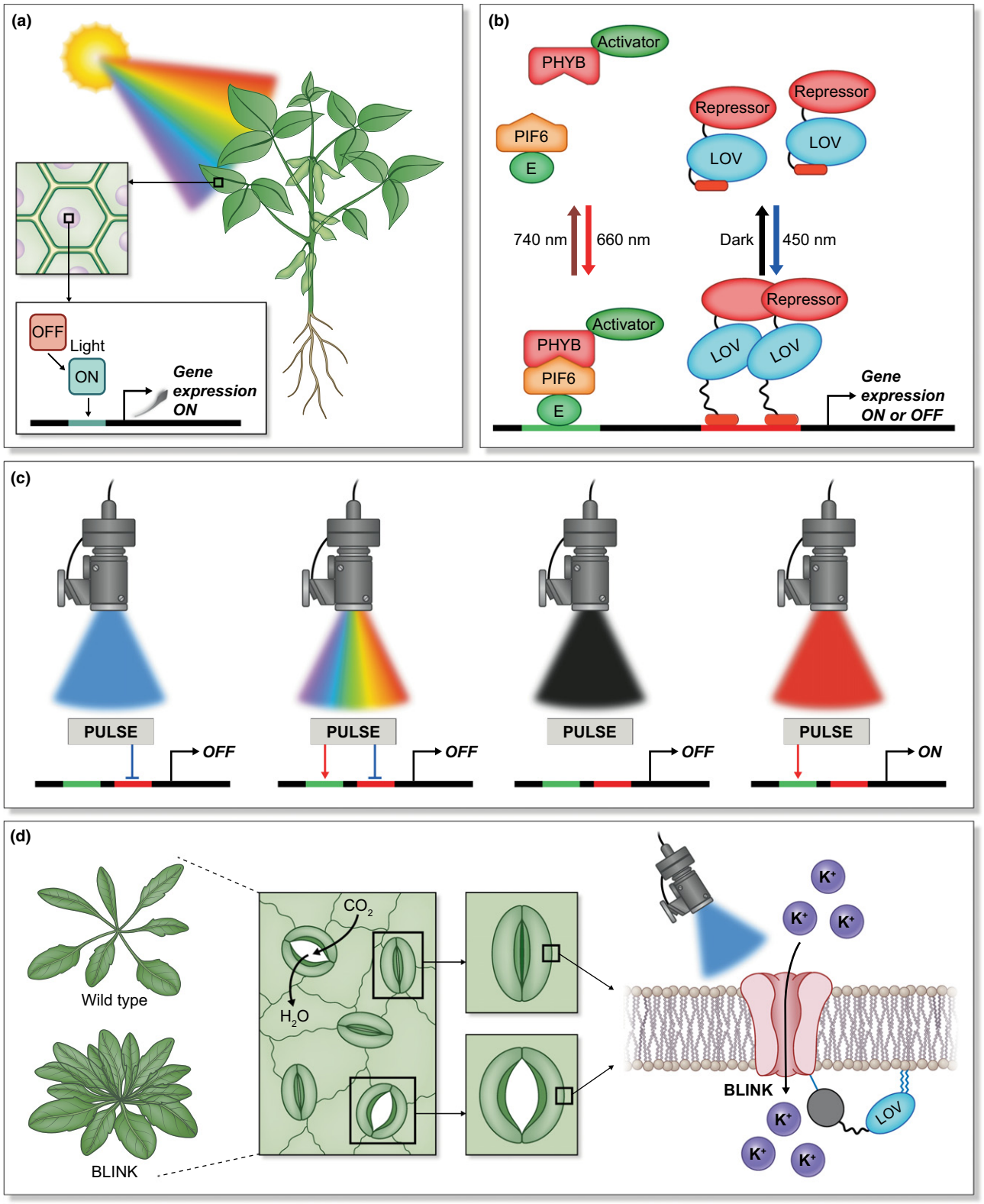

Fig. 3 Application of the first optogenetic tools in plants. (a) A plant's need of white light for growth poses intrinsic experimental limitations for implementing optogenetics, as any photoswitch introduced will be active under ambient light. (b, c) Design principle and mode of function of plant usable light-switch elements (PULSE), an optogenetic tool for the control of gene expression in plants in the presence of ambient light, by combining two photoswitches, one based on the bacterial blue light-receptor EL222, which turns gene expression off under blue light ($Blue_{off}$), and a second one activating expression under red light (Red_{On}) engineered from PHYB. The system is active only in the presence of monochromatic red light, and is off in any other light condition, namely ambient/white light, blue light or in the dark. (d) Optogenetic optimization of plant physiology. Blue light activates BLINK1, leading to an increased K^+influx in guard cells. This enhances stomatal opening kinetics contributing to improved CO_2assimilation while H_2O status is preserved, leading to an overall increase in biomass.

and is capable of conducting both inward and outward currents depending on the K^+ concentration gradient across the membrane. This tool, along with its successor BLINK2 (Alberio *et al.*, 2018), has been used successfully to inhibit neural function in zebrafish, mice and rats (Cosentino *et al.*, 2015; Alberio *et al.*, 2018). Recent work by Papanatsiou *et al.* (2019) has shown that BLINK1 can improve stomatal movements in Arabidopsis when expressed in the guard cells surrounding the stomatal pore. PHOTs trigger blue light-induced stomatal opening by activating the plasma membrane H^+-ATPase, the activity of which promotes K^+ uptake that is required to drive changes in guard cell volume and turgor. Guard cell-specific expression of BLINK1 works on top of this process by using the same wavelength dependency of PHOTs to enhance stomatal opening kinetics by adding another light-driven K^+-uptake pathway. BLINK1 was also found to increase the rate of stomatal closure in darkness (Papanatsiou *et al.*, 2019), presumably by promoting K^+ efflux as a consequence of its slow recovery kinetics (Cosentino *et al.*, 2015). As a result, BLINK1-expressing plants produced more biomass, especially under fluctuating light conditions typical of outdoor growth, because their accelerated stomatal responses synchronize better with the changing light environment to enhance carbon assimilation while preserving water status (Papanatsiou *et al.*, 2019). Moreover, with this approach, unwanted stimulation of endogenous photoreceptor signalling becomes no longer an issue, as the same light quality is used to drive both stomatal opening and BLINK1 activation (Fig. 3d).

V. Channelrhodopsin-based optogenetics in plants

The algal retinal-binding protein, channelrhodopsin-2 (ChR2), is the most commonly used optogenetic tool in the neurosciences (Box 1). ChR2 allows the influx of Ca^{2+} and Na^+ when illuminated by blue light, resulting in membrane depolarization and neural activation. ChR2, like other algal and microbial opsins, is fully functional in mammalian cells where its all-trans-retinal chromophore is in plentiful supply. Supplementation with exogenous retinal has now been successful in fully reconstituting a functional derivative of ChR2 when expressed transiently in *N. benthamiana* or stably in transgenic Arabidopsis, leading to the first demonstration of ChR2-based optogenetics in plants. By studying ChR2-mediated membrane depolarization, Reyer *et al.* (2020) were able to show that the plasma membrane H^+-ATPase is a major driver of membrane repolarization. This work gives a tantalizing glimpse of the potential for opsin-based tools (Box 1) in controlling plant ion transport. A requirement for cofactor addition, however, limits the implementation of these tools *in vivo*. Ongoing work towards engineering the biosynthesis of all-trans retinal in plants (G. Nagel, pers. comm.) will thereby facilitate the use of opsin-based tools for controlling ion fluxes, cellular metabolism and physiology by harnessing their full potential (Box 1).

VI. Optical tuning to modulate photoreceptor sensitivity

Optogenetic tools deactivate in darkness (from seconds to hours, depending on the photosensor) by a process called thermal reversion. Slowing down thermal reversion (slow cycling) through protein engineering can increase photoproduct lifetime, thereby enhancing photosensitivity, whereas the opposite can be achieved when thermal reversion is accelerated (fast cycling). These principles can be used to fine-tune the photocycle kinetics and sensitivity of optogenetic tools (Ziegler & Moglich, 2015). This approach has also been applied to modify plant photoreceptor performance. For example, slow-cycle tuning of ZTL impacts the circadian period in Arabidopsis by altering the rate of clock-component degradation (Pudasaini *et al.*, 2017). A similar strategy has been used more recently to create slow-cycling variants of Arabidopsis PHOTs. Plants expressing these engineered photoreceptors exhibit more rapid and robust chloroplast movement responses and improved leaf positioning and expansion, leading to enhanced biomass accumulation under light-limiting conditions (Hart *et al.*, 2019). While this approach is technically not optogenetics *per se*, these findings demonstrate its feasibility to alter plant photoreceptor for benefits in growth. Changes in temperature can also impact the rate of thermal reversion and impact photoreceptor performance in plants (Jung *et al.*, 2016; Legris *et al.*, 2016; Burgie *et al.*, 2017; Fujii *et al.*, 2017).

VII. Conclusions and outlook

Plant optogenetics is still in its infancy and awaits extrapolation by incorporating additional approaches from the mammalian field, including optical control of kinase regulation, protein localization and protein stability (Box 2), and the use of other opsin-based tools, besides ChR2, to manipulate membrane potential changes and cellular signalling (Box 1). However, implementing such tools will be challenging as this may require additional protein engineering strategies to circumvent issues that could impede their functionality or provoke unwanted side-effects in plant cells. These include chromophore availability (e.g. a need for engineering cofactor production within plant cells), efficient expression and localization, as well as minimizing any interference between plant-derived tools and endogenous light signalling pathways. Given the high quantitative modulation, spatiotemporal resolution and the reversible control capabilities provided by optogenetic tools, we envisage that combinatorial approaches such as those used in PULSE will facilitate the targeted manipulation and study of biological processes in light-grown plants. Native and synthetic promoters (Ali & Kim, 2019) could also be used to drive tissue-specific and development regulation of optogenetic tools in plants, whereas multichromatic, independent and orthogonal control over the expression of several traits (multiplexed) will provide new ways to interrogate regulatory networks noninvasively, and with unmatched temporal and quantitative control. A generalized application of engineering strategies integrating mathematical models will also aid the optimization and customization of photoswitches for their dynamic properties, functionalities and cell/organism of choice.

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