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Optogenetic Methods in Plant Biology

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Abstract

Optogenetics is a technique employing natural or genetically engineered photoreceptors in transgene organisms to manipulate biological activities with light. Light can be turned on or off, and adjusting its intensity and duration allows optogenetic fine-tuning of cellular processes in a noninvasive and spatiotemporally resolved manner. Since the introduction of Channelrhodopsin-2 and phytochrome-based switches nearly 20 years ago, optogenetic tools have been applied in a variety of model organisms with enormous success, but rarely in plants. For a long time, the dependence of plant growth on light and the absence of retinal, the rhodopsin chromophore, prevented the establishment of plant optogenetics until recent progress overcame these difficulties. We summarize the recent results of work in the field to control plant growth and cellular motion via green light–gated ion channels and present successful applications to light-control gene expression with single or combined photoswitches in plants. Furthermore, we highlight the technical requirements and options for future plant optogenetic research.

Contents

1. THE HISTORY AND AIMS OF OPTOGENETICS

Optogenetics is a very simple concept: A heterologously expressed (i.e., genetically encoded) protein is activated by light (*opto-*), which leads to activity changes in cellular processes, from within individual cells up to intact organisms. The name optogenetics was first proposed in 2006 for the light induction of action potentials in nerve cells, following depolarization via several different approaches, of which Channelrhodopsin-2 (ChR2) (105) was the most successful by far (93). At that time, genetically encoded green fluorescent protein (GFP) and the whole family of derivatives and fusion constructs were already well established. While these are optogenetic sensors, channelrhodopsins are optogenetic actuators. ChR2 is a light-gated cation channel that was discovered during basic research on hypothetical light-sensitive membrane proteins from microbes and was characterized in 2003 (105) via heterologous expression in animal and human cells (103, 104). The potential of ChR2 as a light-sensitive actuator or optogenetic tool was immediately recognized, and five independent studies with animal cells or live animals followed quickly, showing light-activated stimulation of neurons and muscle cells (11, 13, 67, 87, 102).

A quick reminder about rhodopsins: Only after covalent binding of the chromophore retinal (vitamin A aldehyde) to the protein (opsin) does it absorb visible light and become photoresponsive. According to convention, the holoprotein is then called rhodopsin. Following the successful application of channelrhodopsin variants, the optogenetic field grew rapidly (60), and additional rhodopsins were used as optogenetic tools, including light-activated ion pumps and

anion-specific light-sensitive ion channels. It became obvious that animal cells like *Xenopus laevis* oocytes or human embryo kidney 293 (HEK293) cells contain enough retinal to obtain a distinct light-induced response, but a strong amplification of the signal was observed upon addition of retinal to the medium during expression (103, 104, 105). Later experiments with ChR2 in live animals confirmed this observation, with good responses in chicken or mouse brains without any retinal addition, whereas for light-responses of transgene *Caenorhabditis elegans* (102) or *Drosophila melanogaster* (132), retinal feeding was essential. For *Drosophila*, it could be shown that a mutant ChR2 (XXL) already works very well with low endogenous retinal amounts (29). As we explain below, the lacking cofactor retinal is an even greater problem when aiming at opsin-based optogenetics in botanical research, for example, with the model plants *Arabidopsis* or tobacco.

In addition to the light control over ion fluxes across membranes, which has extensive applications in neurobiology and vision restoration approaches in animal systems, there is a second large group of optogenetic strategies currently engineered to advance cell biology studies and biomedical applications. The so-called optogenetics 2.0, non-rhodopsin light-regulated synthetic molecular devices, provide unprecedented and unmatched quantitative and spatiotemporal resolution and are revolutionizing the way we can experimentally perturb and analyze the dynamics of subcellular processes (37). Optogenetics 2.0 relies on the engineering of microbial and plant photoreceptors to transduce information in the form of photons into a molecular function, mediated, for example, by a change in protein conformation or enzymatic activity (27), which is, in turn, used to control a wide range of cellular processes (80, and see **https://www.optobase.org/**). These range from tools to control gene expression, RNA and protein stability, kinase and cell receptor activity, subcellular localization of proteins and organelles, and even light-controlled synthetic biomaterials, among other processes. Since the establishment of the first tools over 20 years ago, namely the introduction of a red light gene-expression switch in yeast cells in 2002 (135) and the heterologous expression of photoactivated adenylyl cyclases (PACs) in oocytes and mammalian cells for the production of the second messenger cAMP in 2007 (131), the field has quickly advanced. Hundreds of applications in microbial, yeast, and animal systems have been reported, and the approaches are starting to take root in plants (23, 162). There is already a huge bank of engineered photoreceptors of bacterial, fungal, and plant origin that represent the natural diversity of photobiological properties, namely, color and wavelength inducibility, light sensitivity and the kinetics of activation and reversion, and the molecular mechanism of function, that is, light-induced conformational change; enzymatic activity control; or hetero-, homo-, and oligodimerization. Depending on the application, the user can now select from the database the photoreceptor or already engineered photoswitch and plug it into the effector/output domain to control the cellular process of choice. Among the dozens of microbial and plant photoreceptors used to engineer optogenetic switches, the most representative families and wavelengths include UV RESISTANCE LOCUS 8 (UVR8) [ultraviolet B (UV-B) light], light-oxygen-voltage (LOV) and blue light–using FAD (BLUF) domains and cryptochromes (CRYs) (blue light), cobalamin binding proteins (green light), cyanobacteriochromes (green to far-red light), and phytochromes (red to far-red light). The photoreceptors use different covalently or noncovalently bound chromophores, including flavins [flavin adenine nucleotide (FAD), flavin mononucleotide (FMN)], linear tetrapyrroles, and cobalamin derivatives, which need to be naturally present in the target organism or added to the growth medium. The portability of these approaches is not yet straightforward; however, the required engineering and customization processes can be facilitated with the aid of mathematical modeling. Yet it seems that in plants, the requirement for light for growth precludes the widespread implementation of these types of tools (23). Nevertheless, a first tier of reports describes successful strategies for plant optogenetics (see Section 4).

2. LIGHT REQUIREMENT FOR PLANT GROWTH

Thermonuclear reactions within the sun convert mass into energy, which is described by the famous equation of Albert Einstein ($E = mc^2$). The energy, in the form of electromagnetic radiation of ~200–2,500 nm, reaches the earth by wave motion, while only a narrow range of this spectrum (**Figure 1**), called white light (380–750 nm), is visible to the human eye. Wavelengths <290 nm are filtered out by the stratospheric ozone layer, and only a little >1,000-nm radiation reaches

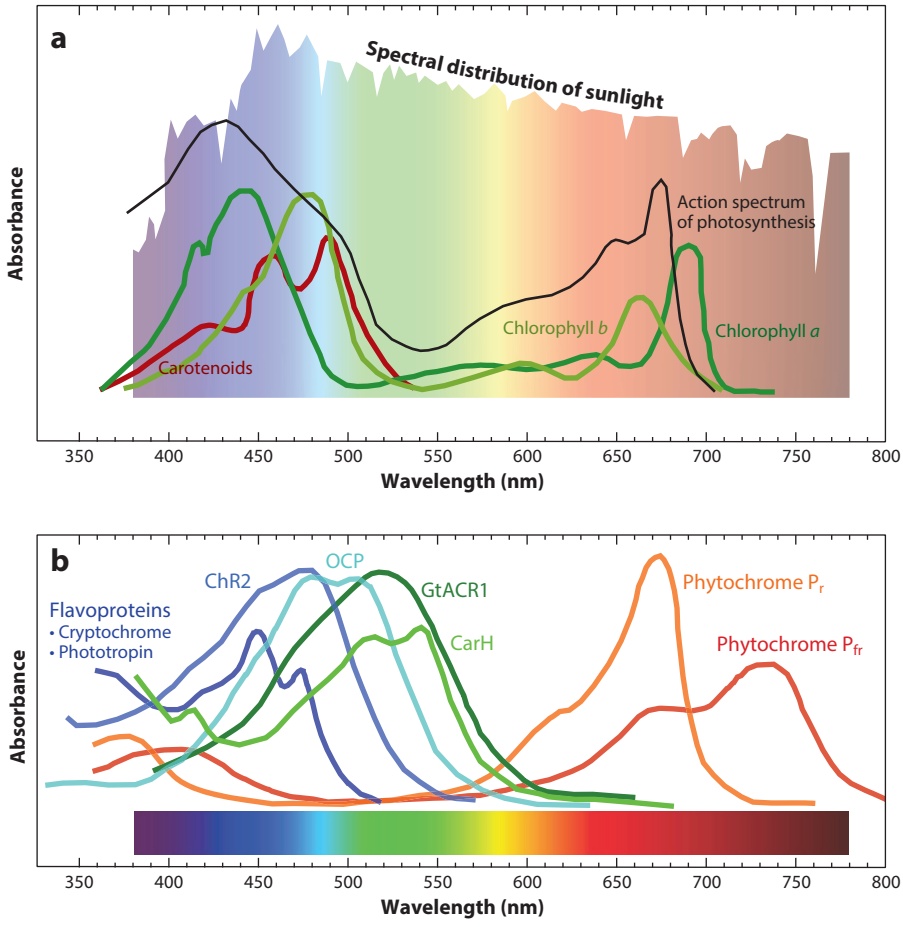


Figure 1

Absorbance spectra of photosynthesis pigments and plant and nonplant photoreceptors. (*a*) The action spectrum of photosynthesis (*black line*) matches well the absorption spectrum of the carotenoids (*red line*) and the chlorophylls (Chlorophyll *a*, *dark green line*; Chlorophyll *b*, *light green line*). The relatively low absorption and photosynthetic activity in the green wavelength spectrum is remarkable. (Spectra in accordance with Reference 71.) The height of the color band in the background corresponds to the relative intensity of the sunlight along the visual spectrum [adapted from Reference 12 (CC BY 4.0)]. (*b*) Absorption spectra of plant photoreceptors such as the P_{fr} (*red*), the P_r (*orange*), and the flavoproteins (*blue*), which include the cryptochromes and phototropins. The plant photoreceptors absorb very little in the green wavelength spectrum, which makes the use of optogenetic tools with partial or maximal absorption in this range ideal. Rhodopsins such as ChR2 or ACR1, as well as OCP or the light-regulated transcription factor CarH, have already been used as optogenetic tools in plants. Abbreviations: ACR1, Anion Channelrhodopsin-1; ChR2, Channelrhodopsin-2; OCP, orange carotenoid protein; P_{fr}, active phytochrome; P_r, inactive phytochrome.

the surface of the earth due to low solar emission and high atmospheric water absorption of this spectrum (153). As a result, the spectral distribution of sunlight reaching the earth is not uniform but curved with a maximum intensity in the blue range (12) (**Figure 1*a***).

The conversion of sunlight into chemical energy represents the origin of all energy for life on this planet and is characteristic of photoautotrophic organisms. From the wavelength spectrum reaching the earth's surface (**Figure 1*a***), plants photosynthetically absorb only a small portion to produce chemical energy while maintaining the earth's atmospheric oxygen content as a by-product. The action spectrum of photosynthesis (**Figure 1*a***) shows that a high proportion of blue and red light is photosynthetically used by plants (124). The light absorption takes place mostly in leaves, through the pigments chlorophyll *a* and *b* and carotenoids (**Figure 1*a***) in the thylakoid membranes of chloroplasts, where the light-harvesting complexes are located. In the green and yellowish part of the sunlight spectrum, much less light is absorbed (**Figure 1*a***), which is why plants appear green.

3. LIGHT-TO-SIGNAL CONVERSION VIA PHOTORECEPTORS

Not only is light used to convert carbon dioxide and water into sugars by photosynthetic microorganisms and plants, but visible radiation also gathers a variety of information allowing organisms to control metabolism, photoperiodicity, phototaxis, and development. Several classes of photoreceptors have been identified that absorb light to orchestrate plant light responses (41, 96, 110). The perception of light by the different photoreceptors is wavelength specific (**Figure 1*b***); however, the majority of light absorption through photoreceptors in plants occurs in the blue, UV, and red/infrared ranges, while the green spectrum is largely omitted (**Figure 1*b***). In part, light signaling by these photoreceptors in plants may be problematic for the application of optogenetic tools in plants, but, similar to numerous nonplant photoreceptors, they can also be turned into optogenetic tools through clever design. Which photoreceptors should be considered for plant optogenetics, which mechanisms underlie photoperception, and how light is mechanistically translated into cellular signaling is briefly discussed below.

UVR8, identified in *Arabidopsis*, is a photoreceptor that absorbs UV-B radiation through a tryptophan cluster within the protein (Trp233, Trp285, and Trp337), thereby becoming activated via dimer-to-monomer switching (21, 30, 156). The monomeric form of UVR8 subsequently interacts with CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) or several other proteins, including transcription factors, to control photomorphogenic development in plants (38, 116). While the currently available UVR8-based optogenetic tools rely on the interaction with COP1, its binding with other proteins could possibly allow the design of diverse UV-light switches. The use of UVR8 as an optogenetic tool could find wide application (100) because it does not require an exogenous chromophore; however, some unfavorable UVR8 features need to be considered carefully. Advantages and disadvantages of a UVR8-based optogenetic tool are excellently discussed in a recent review (109).

The known blue light receptors such as phototropin 1 and 2 or the CRYs belong to the flavoproteins and need a cofactor/chromophore for light absorption (20, 57, 96, 151). The characteristic absorption of flavin-containing proteins is in the blue light spectrum (between 400 and 500 nm) (**Figure 1*b***). CRYs and phototropins have FAD, and phototropins have FMN as chromophores. Both FAD and FMN bind to a LOV domain to form the functional chromophore. LOV domains represent light-absorbing modules that evolved in multiple kingdoms to sense blue light (46, 62) and regulate a vast amount of different light responses (46). Together with BLUF domains, which are prevalent in prokaryotes, LOV domains are blue light–regulated photoswitches ideally suited for optogenetic approaches. Blue light optogenetic photoswitches

based on LOV and BLUF domains have become popular lately outside of plant science in model organisms/systems devoid of blue light sensing (133). LOV and BLUF domains both generally consist of short (100–140 amino acid residues) α/β-modules and undergo conformational changes upon blue light perception, while the photophysical mechanisms differ between them (40). The LOV domain undergoes a reversible photocycle that includes the formation of a thiol adduct between a conserved cysteine residue in the protein and the chromophore. The BLUF domain does not undergo such a covalent intermediate step, but blue light excitation rearranges the hydrogen bond network (22). The LOV-based photoreceptors are usually composed of modular sensor and effector domains whose interactions are mediated by the α-helical linker between them (62, 118). By contrast, the BLUF photoreceptors mostly mediate their physiological response through a light-induced protein–protein interaction rather than an effector-induced activity change (40).

The first photoreceptors ever to be identified were the phytochrome (*phyto* meaning plant; *chrome* meaning color) red light receptors. Plant phytochromes absorb light in the red (650–670-nm) and far-red (also called near infrared; 705–740-nm) spectrums (**Figure 1*b***) to control cellular and developmental processes by sensing the ratio between the two wavelengths (18). Red light absorption by the inactive phytochrome (P_r) ground state induces a *Z*/*E* isomerization at the C–D methine bridge around the C15=C16 bond of the chromophore (145). This leads to structural changes and protein–protein interactions, inducing the movement of the active phytochrome (P_{fr}) to the plant cell nucleus, which is considered the key step in phytochrome signaling (74). The P_{fr} form of phytochrome A (PHYA) or PHYB binds hundreds of proteins, including the phytochrome-interacting factors (PIFs) known to be transcription factors to initiate the light response (113). Far-red light and darkness convert the molecule back into the P_r form. Thus, phytochromes are reversible red/far-red light–activated molecular switches. The chromophore of canonical plant phytochromes is phytochromobilin, and that of nonplant phytochromes is phycocyanobilin or biliverdin. The open-chain tetrapyrrole chromophores are covalently bound to different cysteine residues of the apoprotein (82). Because these tetrapyrroles are not present in mammalian cells, they must be supplemented to the medium to form functional phytochromes for optogenetic approaches (99). However, this also has advantages since the absence of the chromophore allows the specimens to be handled without the need for special light conditions prior to the addition of the cofactor.

In opsins, another class of photoreceptors that are not present in plants (5), the chromophore retinal is readily available in animals but not in plants, even though the precursor provitamin A (carotenoid) is abundant in plants (161). Opsins bind the chromophore retinal covalently via a protonated Schiff base with a perfectly conserved lysine residue to form functional rhodopsins (137). While animal (type 2) rhodopsins represent the visual pigments in the eyes of vertebrates, invertebrates, and insects, light-sensing microbial (type 1) rhodopsins have diverse functions (137, 161).

It is remarkable that the same retinal chromophore in rhodopsins can lead to a very broad range of absorption maxima, which lie in the entire visible light spectrum of 300–700 nm (36). Both the protonation status of the chromophore and the protein environment with charged, uncharged, or aromatic amino acids play a crucial role for color tuning (70, 89, 142).

Aside from photosynthesis to convert light into chemical energy, rhodopsins are the only other means of light-dependent chemical energy production. Rhodopsins allow a diverse array of Bacteria and Archaea to live photoautotrophically (19, 58, 90). Proteorhodopsins are ubiquitous in bacterioplankton, and studies on the ecological significance of global light-driven energy production demonstrated the important role of these H^+-pumping rhodopsins for the conversion of light to chemical energy in the oceans (115). There is growing evidence that H^+-pumping rhodopsins

allow an increase in proton motive force, which results in a higher membrane transport capacity and/or H^+-fueled ATP-synthesis (47, 91, 98).

In addition to the H^+-pump rhodopsins, rhodopsins with cation and anion channel functions play important roles as optogenetic control elements, particularly in neurobiology. The ChR2 and the Anion Channelrhodopsin-1 (ACR1), with absorption maxima in the blue or green wavelength spectrum (**Figure 1*b***), are implemented for the electrical stimulation or inhibition of neuronal networks, respectively (2, 50). Both ChR2 and ACR1 have recently been applied in plants to adjust ion transport for the control of cellular growth and movement (161), which are discussed in Sections 6.1 and 6.2.

In optogenetics, heterologous expression of known photoreceptors is used to induce desired cellular changes in a target organism in a noninvasive way by means of light (**Figure 2**). While microbial rhodopsins can optogenetically be used to induce light-dependent ion movements, blue light-perceiving CRY, LOV, and BLUF proteins or domains thereof or red/infrared-sensing phytochrome (PHY) domains are (non-rhodopsin) optoswitches to control, for instance, protein targeting, protein activity, or gene expression (**Figure 2**). In protoplasts, leaves, or intact plants, transient or stable expression of optotools can be accomplished with plasmid DNA or agrobacterium-mediated transfection, respectively (**Figure 2**). Because of the predominant use of blue and red light for photosynthetic energy production and control of physiological processes in plant cells, green light, which is the least absorbed, is ideal for optogenetic control, for example, by ACR1 (160–162) (**Figure 1*a*,*b***; see Section 6).

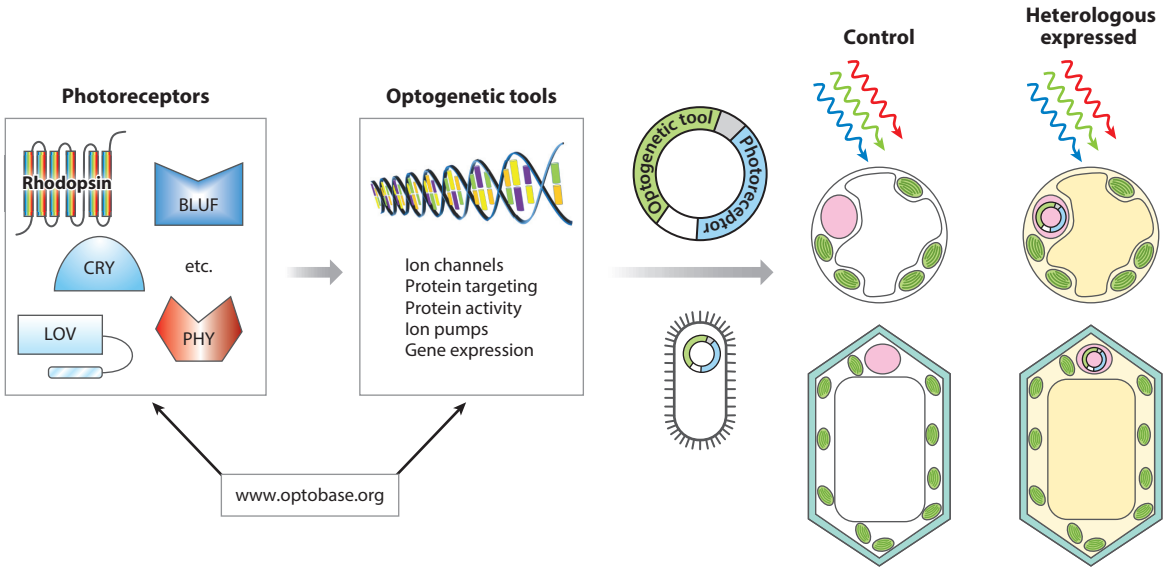


Figure 2

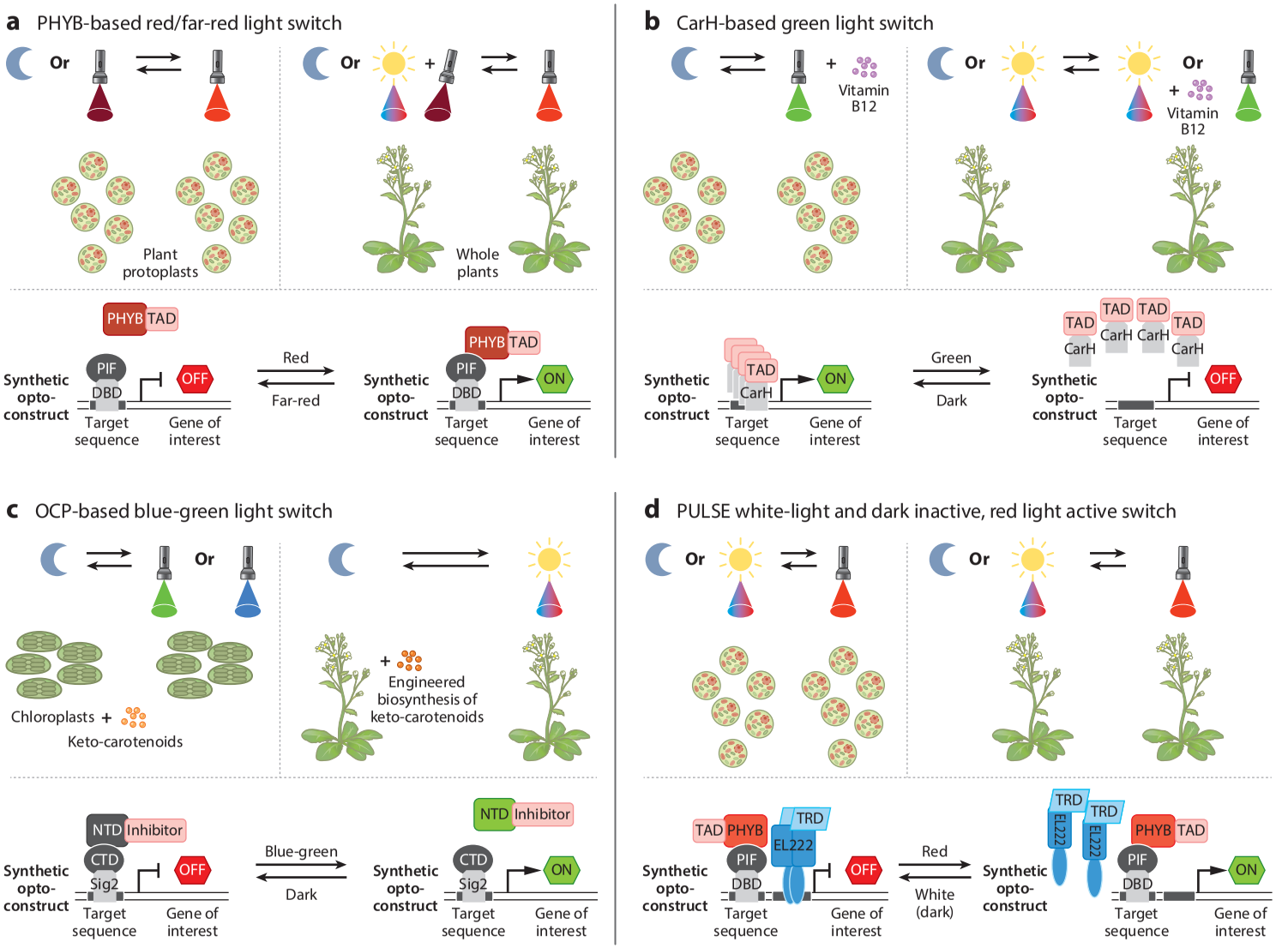
Flow chart showing how to apply optogenetics in plants. A list of which non-rhodopsin-based optogenetic switches can be used for which purpose and at which wavelength of activation/inactivation can be found in the OptoBase database (**https://www.optobase.org/**). The optoswitches can be used to induce light-induced changes in, for example, protein targeting, protein activity, or gene expression. The optogenetic switches can include blue light–sensitive domains, such as LOV, CRY, or BLUF, or red/infrared light-switchable domains, such as PHY. Membrane-located rhodopsins are used as light-dependent ion channels or pumps to induce electrical changes or ion fluxes (e.g., Ca^{2+}). These genetically encoded tools can be introduced into appropriate vectors for the transformation of protoplasts or intact plants in order to initiate light-controlled processes. Abbreviations: BLUF, blue light–using FAD; CRY, cryptochrome; LOV, light-oxygen-voltage; PHY, phytochrome.

4. NONOPSIN-BASED PLANT OPTOGENETICS FOR THE CONTROL OF CELLULAR PROCESSES

A major goal of using optogenetics is to be able to switch signaling and metabolic networks on and off with adjustable kinetics, reversibility, and quantitative and high spatiotemporal resolution, thereby facilitating fundamental research, for example, developmental studies requiring the temporal activation of signaling components in defined subpopulations of cells. However, plants need light for growth and to make developmental and life fate decisions, as described in Section 2. This poses an intrinsic experimental limitation on a widespread implementation of optogenetic approaches in plants, as most optoswitches will be inadvertently activated under normal light growth conditions. The sunlight spectrum impacting the earth's surface logically comprises the wavelengths that activate the photoreceptors of organisms adapted to sense exactly those colors. We describe here the first strategies employed to engineer optogenetic tools for use in plant cells and whole plants for the control of gene expression, which partially or completely overcome this experimental and technical challenge. For this, creative approaches have to be implemented, including the exploitation of the photoswitchability of photoreceptors of the phytochrome family, the use of wavelengths not frequently used for plant signaling (e.g., green), or the combining/multiplexing of optoswitches.

4.1. Dual Switchability: Phytochrome Family–Based Tools

As described in Section 3, most photoreceptors are activated upon absorption of a photon matching a more or less broad absorption peak and revert to the basal state by thermal relaxation (i.e., dark reversion). However, phytochromes are unique in the sense that they shuttle between two photostates, having two partially overlapping absorption spectra (15). This photobiological property can be exploited in optogenetics for the targeted activation or inactivation of the photoswitch by using two wavelengths of light, for example, in the case of plant phytochromes, red (~660-nm maximum) and far-red (~760-nm maximum) light. The first optoswitch introduced into plant cells is a synthetic split transcription factor module engineered from *Arabidopsis thaliana* PHYB and its interactor PIF6 (101) (**Figure 3*a***). In brief, a truncated version of PHYB (N-terminal 660 amino acids) was fused to a viral transactivator VP16 and a nuclear localization sequence (NLS), and the first 100 amino acids of PIF6 were fused to a DNA-binding domain based on the macrolide repressor protein from *Escherichia coli* (E protein). Illumination with red light leads to heterodimerization of the components, reconstructing a functional transcription factor that drives expression from a synthetic promoter comprising the specific binding site of the E protein and a viral minimal promoter. Shining far-red light terminates gene expression (**Figure 3*a***). The photoswitch was first implemented in *Nicotiana tabacum* and *Arabidopsis* protoplasts, fully characterized in terms of light- and intensity-dependent activation/inactivation dynamics, showing reversibility and up to 400-fold activation rates (101, 107). The system was used to fine-tune a plant signaling pathway, namely auxin signal relay in *Arabidopsis* protoplasts. Red light allowed control over expression or downregulation of the levels of the auxin receptor TIR1, which led to hyper- and hyporegulation of the auxin response, respectively, as monitored using a quantitative genetically encoded auxin biosensor (152). This is a proof-of-principle demonstration that, by profiting from the combination of the quantitative control capabilities of optogenetic tools with a quantitative readout/output module, it is possible to interrogate and manipulate a signaling network to better understand the mechanistic regulatory principles involved (101). This switch was also introduced into *Physcomitrium patens* to control the recombinant expression of a human growth factor (VEGF) (101). The switch can in principle be used in whole plants grown under cycles of dark/white light, provided that a low dose of supplemental far-red light is provided to keep the system in the off



(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

The illumination conditions and molecular mechanisms of functions of gene-expression optoswitches implemented in plant cells and whole plants. (*a*) Phytochrome-based red/far-red; (*b*) CarH-based green; (*c*) OCP-based blue-green; and (*d*) PULSE white light–blind, red light–inducible gene expression switches. Depicted are the experimental illumination conditions and activation/inactivation states for the optogenetic systems when introduced into plant protoplasts or whole plants (*top* of each panel) and the molecular mechanisms of function with their wavelength-dependent changes in activity (*bottom* of each panel). The cofactors are indicated if exogenous supply or pathway engineering is needed. Detailed descriptions of the molecular mechanisms of function in panels *a*, *b*, *c*, and *d* are in Sections 4.1, 4.2, 4.3, and 4.4, respectively. Sunlight represents white light. Abbreviations: CarH, *Thermus thermophilus* AdoB12-binding transcription factor; CTD, C-Term of OCP; DBD, DNA-binding domain; EL222, *Erythrobacter litoralis* LOV domain–containing helix-turn-helix transcription factor; NTD, N-term of OCP; OCP, orange carotenoid protein; PHYB, phytochrome B; PIF, phytochrome-interacting factor; PULSE, plant-usable light-switch element; Sig2, plastidial Sigma factor 2; TAD, transcription activation domain; TRD, transcription repression domain.

state. There is the risk of potential effects on endogenous signaling pathways by having a slightly modified white light growth spectrum that requires the proper control experiments.

4.2. Profiting from the Green Light Signaling Gap of Plants: The CarH System

An interesting strategy for optogenetic control in plants is to exploit small windows in the white light spectrum, that is, wavelengths that plants do not use as key signaling cues for development and growth. The most straightforward strategy is to engineer receptors in the green/yellow region (500–600 nm). However, there are not many photoreceptors available that mediate protein–protein interactions to allow the development of an optogenetic expression switch. Chatelle et al. (16) engineered CarH, a transcription factor of the bacterium *Thermus thermophilus* that is naturally activated by green light (69) (**Figure 3*b***). The switch comprises CarH fused to a transactivator (VP16). In the basal state (dark), CarH is a tetramer that binds to the CarO DNA sequence placed upstream of a minimal promoter, controlling the expression of any gene cloned downstream to it. Irradiation with green light breaks the tetrameric structure, and the CarH monomers disassemble from the promoter, terminating gene expression (**Figure 3*b***). The system is activated with low intensities of 525-nm light (5 μE m^2 s^{-1}) in *Arabidopsis* protoplasts. Use in whole plants is limited by the fact that CarH incorporates adenosylcobalamin (AdoB12, a derivative of vitamin B12) as an unusual chromophore. As plants do not produce vitamin B12, the chromophore should be infiltrated into the tissues in order to obtain the active, holo form of the photoreceptor. This is an experimental limitation that can only be eventually overcome upon engineering of the AdoB12 biosynthetic multienzyme pathway into plants. Interestingly, however, the advantage of the need to add the chromophore for activity is that the system would remain inactive during plant growth regardless of the illumination conditions and would only become primed for activity upon exogenous AdoB12 addition.

4.3. Orange Carotenoid Protein: Green-Blue Switch in Plant Chloroplasts

The orange carotenoid protein (OCP) family of cyanobacterial photoreceptors has been recently engineered for plant optogenetics. Irradiation of OCPs with blue-green light leads to a conformational change, spatially separating the N- and C-terminal domains. The protein returns to the basal state in the dark by thermal relaxation (77). The molecular mechanism was recently exploited to generate a heterodimerization switch (83, 97) (**Figure 3*c***). Two proteins/domains are each fused to either the N- or C-terminals of OCPs, which interact in the dark, bringing them in close spatial proximity. Blue-green light terminates the interaction (**Figure 3*c***). Piccinini et al. (114) integrated this molecular principle to engineer a photoswitch to control protein–protein interaction/heterodimerization in *Arabidopsis* chloroplasts. As OCPs use a keto-carotenoid as a

noncovalent bound chromophore that is not produced in plants, the authors first engineered keto-carotenoid synthesis, namely canthaxanthin or echinenone conversion from β-carotene by introducing a bacterial β-carotene ketolase (crtW) into chloroplasts (**Figure 3*c***). The optogenetic tool was first used to reconstruct a split luciferase and afterward further customized to control gene expression in chloroplasts. For the latter, the two components of the photoswitch were fused to the plastidial Sigma factor 2 and the T4 phage antisigma factor AsiA. Illumination with high green light intensities (350 $\mu E\ m^2\ s^{-1}$) leads to activation of gene expression. The OCP-based optogenetic tool works in proof-of-principle experiments in chloroplasts of *Arabidopsis* protoplasts and whole plants, opening up useful applications for plastidial-targeted manipulations, for example, gene expression and enzyme activity control. For potential users, it is worth noting the need to use keto-carotenoid-producing engineered plants and that the optoswitch will be active under white light growth conditions. Certain algae naturally produce keto-carotenoids, so the system might be readily applicable.

4.4. A Switch for Plants Growing Under Normal Light Conditions—Cycles of Dark/White Light: PULSE

As discussed above, due to the natural photobiological properties of photoreceptors, it is cumbersome (requiring the manipulation of light conditions or chromophore availability) to achieve an optogenetic system to be used in plants growing under normal photoperiods, that is, alternating cycles of darkness and white light. An alternative approach is to implement synthetic biology strategies to engineer a system that can perform as experimentally required. There is already a plethora of photoswitches sensitive to different wavelengths of light and capable of being coupled to the regulation of almost every biological process that can be multiplexed for simultaneous use and therefore regulation. In order to develop a light-controlled gene expression switch blind to white light, and only activated on demand with a given discrete wavelength of light, Ochoa-Fernandez et al. (106) integrated two different switches controlling a synthetic composite promoter, thereby engineering a tool termed plant-usable light-switch element (PULSE) (**Figure 3*d***). The first optomodule is a blue-off switch engineered from EL222, a bacterial LOV-domain containing transcription factor activated by blue light, fused to a transrepressor (SRDX). The second is the red-on PHYB-based switch described in Section 4.1. Under white light, both switches will be bound to the promoter (blue and red light are represented in white light) and the blue-off switch will dominantly repress gene expression (**Figure 3*c***). In the dark, no switches will be bound and gene expression will also be off. Only in the presence of monochromatic red light, which is a condition that never exists in nature, will gene expression from the synthetic promoter ensue (**Figure 3*c***). This allows growing plants under normal conditions in plant chambers or greenhouses without the risk of inadvertent, undesired activation of the optogenetic device. The system is active in transient expression experiments in *Arabidopsis* protoplasts and *Nicotiana benthamiana* leaves and in stable *Arabidopsis* transformants. PULSE can be used to control signaling and metabolic networks and readily be integrated with CRISPR-Cas-based tools, making it a promising molecular device for fundamental research and biotechnological applications. The system comprises two switches, making it more complex in molecular terms (e.g., promoters, relative expression rates, and resistance markers); therefore, portability into different plants requires some customization.

5. MICROBIAL RHODOPSIN-BASED PLANT OPTOGENETICS

Ion signaling plays vital roles in plants (59), and light-gated ion channels are of great interest, owing to their potential to be used to dissect plant electric, Ca^{2+}, and proton signaling. The

control of plant transpiration is largely determined by ion fluxes in or out of guard cells and the resulting stomatal movements. A synthetic light-regulated K^+ channel, BLINK1, was expressed in *Arabidopsis* guard cells and found to enhance stomatal opening and closing kinetics and improve carbon assimilation upon alternating light conditions (112). BLINK1 was engineered from a viral potassium channel that was linked to a blue light–sensing LOV2 domain (25). The most widely used optogenetic tools for the manipulation of ion fluxes, however, are microbial rhodopsins, which have had an enormous impact in neuroscience and other animal research (51, 123). Rhodopsins function as ion pumps or channels and are routinely used to depolarize/hyperpolarize cells and regulate pH or Ca^{2+} signaling (**Supplemental Table 1**), and they hold great potential for applications in the field of plant physiology research.

Supplemental Material >

5.1. Application of Microbial Rhodopsins in Plants

In contrast to most scientifically used animal cell systems, which are rarely subject to intrinsic photoreceptor regulation, plants require light for growth through photosynthetic carbon gain and control of developmental processes. This requirement has long hampered the use of optogenetic applications in plants. Microbial rhodopsins normally have broad action spectra, and most of them are sensitive to blue and green light (129), which would inevitably lead to optogenetic stimulation using commercially available growth chambers or greenhouses. In plant rhodopsin-based optogenetics, the use of specific red light growth conditions achieved with either white light sources combined with red (>600 nm) filters or illumination with red LEDs (650 nm) is necessary. Using red light for plant growth, optogenetic stimulation in transgenic plants expressing microbial rhodopsins can be prevented while the transgenic tobacco plants grow and develop normally (161).

An important breakthrough for rhodopsin-based optogenetics was the genetic introduction of a bacterial dioxygenase to produce the chromophore retinal in plants (161) (**Figure 4*a*,*b***). The presence and covalent binding of retinal to the opsins are essential for the formation of functional rhodopsins. The synthetic biology approach for in planta retinal production thus overcame some of the biggest problems to apply rhodopsin-based optogenetics in plants, namely chromophore availability.

Despite being integral membrane proteins, subcellular targeting to the plasma membrane of microbial rhodopsins is highly variable and was found to be very inefficient in plant cells. However, there is a large library of microbial rhodopsins available that can be used to screen for variants with better plasma membrane–targeted expression. Modification of the N and C termini of microbial rhodopsins with artificial signal peptides or plasma membrane trafficking motifs was shown to enhance the plasma membrane localization in plant cells significantly (160, 161). Another important aspect to consider is that the presence of retinal is necessary for protein stability and functional expression of microbial rhodopsins (29, 102, 105, 146). The fact that the essential cofactor retinal is missing in plants was very likely the cause of the slow progress in rhodopsin-based plant optogenetics, since in the absence of retinal, degradation of rhodopsin takes place (146).

A powerful channelrhodopsin mutant, ChR2-XXL, with robust expression and enhanced channel activity even under low retinal condition in animal cells (29) was successfully employed in plants (121). Functional ChR2-XXL could be expressed in plants (transient expression in *N. benthamiana* and stable expression in *A. thaliana*) after addition of exogenous retinal (121). However, this approach is not applicable so far with other channelrhodopsin variants, according to our own experience, because without enough retinal, most rhodopsin variants cannot be expressed as well as ChR2-XXL. Another aspect of this approach is that after external application, retinal is not stable, and light exposure will result in retinal oxidation and degradation over time.

Although it can be strongly assumed that no reconstitution of functional rhodopsins is possible in the absence of the cofactor retinal, there were several reports on bacterio-opsin-induced stress

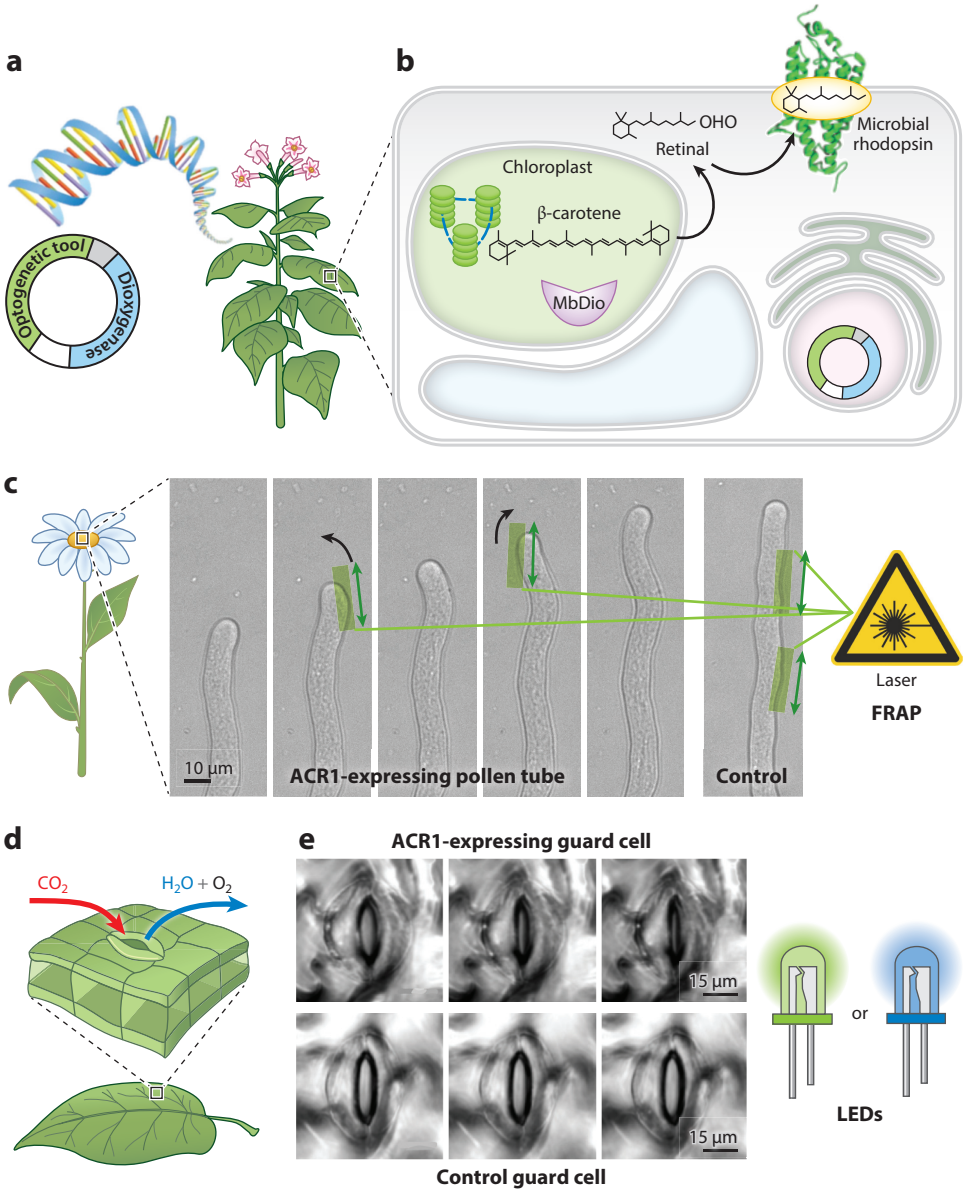


Figure 4

Rhodopsin-based optogenetic applications in plants. (*a*) Transformation of plants with sequences for the rhodopsin-based optogenetic tool and an MbDio that empowers (*b*) the synthesis of retinal from carotenoids in planta. The presence of retinal enables the functional expression of rhodopsins. (*c*) In ACR1-expressing pollen tubes, but not in control pollen tubes, local light application via laser by the FRAP module made it possible to steer growth direction away from the ACR1 activation site. (*d*) Stomata play a major role in the control of leaf transpirational water loss and gas exchange for photosynthesis (panel *d* adapted with permission from **https://evolution.berkeley.edu**, © UC Museum of Paleontology Understanding Evolution). (*e*) Global green or blue light illumination via LEDs allows ACR1-expressing stomata to close but not the control guard cells [panel *e* adapted from Reference 64 (CC BY-NC 4.0)]. Abbreviations: ACR1, Anion Channelrhodopsin-1; FRAP, fluorescence recovery after photobleaching; LED, light-emitting diode; MbDio, marine bacterial dioxygenase.

responses in plants (95, 117, 122). Now that in planta retinal synthesis is established (161), whether the plant responses observed are related to light-driven H^+ pump activity by bacterio-opsin could be confirmed.

5.2. Anion Channelrhodopsin-Induced Depolarization

To overcome the problem of retinal availability in plants, a marine bacterium dioxygenase (MbDio) (76) was recently expressed in plants to produce retinal in vivo (161) (**Figure 4*a*,*b***). MbDio was targeted into the chloroplasts of plant cells after N-terminal fusion with a synthetic chloroplast targeting peptide RC2 (134) (**Figure 4*a*,*b***). The MbDio could convert β-carotene into retinal in the chloroplast, improving the functional expression of the light-sensitive anion channel *Gt*ACR1 (161) and other anion channelrhodopsin members (160) to the plasma membrane. This strategy for in planta production of retinal will possibly allow a huge palette of available rhodopsins to be functionally expressed in plants in the future.

When using green light, which is photosynthetically used the least (**Figure 1*a*,*b***), the optogenetic stimulation of *Gt*ACR1 (50, 52) version 2.0 (ACR1 2.0) induced membrane potential depolarizations of high amplitude in several tobacco cell systems (161). This optogenetic tool was well suited to trigger temporally defined membrane potential changes in pollen tubes (161). This was the first optogenetic demonstration of precise noninvasive spatial and temporal control of the membrane potential by local green laser light stimulation of ACR1 2.0 in plant cells. Local green light illumination at the flanks of the apical dome of pollen tubes enabled guidance of the growth direction (161) (**Figure 4*c***), further substantiating the evidence of the importance of tip anion efflux to control pollen tube growth (55, 56, 61). In guard cells, which are relevant for plant water balance and gas exchange for photosynthesis (**Figure 4*d***), activation of ACR1 2.0 successfully initiated stomata closure (**Figure 4*e***) through a massive efflux of anions that was subsequently followed by a bulk cation efflux caused by depolarization-activated voltage-gated potassium channels (64). Using the optogenetic approach with ACR1 2.0, it was possible to show that an anion channel–induced membrane depolarization is sufficient to initiate stomatal closure (64).

6. FUTURE OPTOGENETIC APPROACHES FOR IN PLANTA USE

Boosted by the discovery of channelrhodopsins, different types of photoreceptors with diverse functions have been used to design light-controllable proteins (7, 37). A large part of these existing tools can be directly used for plant research. We list several examples of established optogenetic tools and fields of applications [e.g., ion signaling; reactive oxygen species (ROS) production; protein relocalization, interaction, and activity; and gene regulation] that are intriguing for plant applications (**Supplemental Table 1**). For a comprehensive list of optogenetic tools and applications outside of plant science, readers can refer to recent reviews (7, 37).

Supplemental Material >

6.1. Light-Controlled Dissection of the Electric, Ca^{2+}, and Reactive Oxygen Species Networks: Is It Possible?

Electric, Ca^{2+}, and ROS signals are early plant reactions in response to various types of stress such as pathogen attack, wounding, water stress, and salt stress, and they are thought to be intertwined (140, 147). An auto-propagating ROS wave (94) was directly linked to the Ca^{2+} wave (45). How these three signals are mechanistically linked to one another is not fully understood since it was not possible in the past to generate these signals individually and sequentially. Optogenetic methods offer the opportunity to identify the role of these signaling components by triggering them individually in a noninvasive manner. Thus, direct manipulation of ROS is of interest in addition to the existing tools that could generate electric (by ACR1 2.0) and Ca^{2+} signals.

There is a growing field of Ca^{2+}-signaling researchers using Ca^{2+} biosensors to investigate the role of Ca^{2+} signals, and the ability to manipulate cellular Ca^{2+} in plants has been sought for a long time. In the animal fields, optogenetic manipulation is widely applied using high Ca^{2+}-conductive channelrhodopsins such as CatCh (79), ChR2-XXM (33, 130), and *Ps*CatCh 2.0 (17). Tools for ROS production such as mini Singlet Oxygen Generator (miniSOG) (119) and miniSOG2 (88) or fluorescence proteins such as KillerRed and SuperNova (108) were used for inducing cell ablation (88, 119), targeted degradation of proteins (48), and modulating activities of channels such as TRPA1 and TRPV1 (68) in animal cells. The utilization of these tools in plants is straightforward, and the beauty of the optogenetic approach for ROS manipulation is that the ROS levels can be fine-tuned by the power and duration of the light input. Optogenetic tools with similar functions but different light sensitivity and kinetics are continuously being developed to achieve precise optogenetic control. For example, the light-gated channel OLF-bP (8) with large conductance and *Ps*CatCh 2.0 (17) with fast kinetics are employed for the generation of Ca^{2+} waves with different amplitudes and time resolutions, respectively. The blue-shifted anion channelrhodopsin *Gt*ACR2 and fast-responsive anion channelrhodopsin ZipACR (52) have been established in plants already to achieve membrane depolarization and manipulation of anion flux (160) with different wavelengths and kinetics.

6.2. How to Break the Code for pH Signaling by Light

The importance to plants of environmental pH sensing was recognized for more than 100 years (26). The regulation of intracellular pH or proton flux as a signal messenger was proposed two decades ago (39, 53). However, only very recent work demonstrated cellular pH to have control of the ABA-signaling pathway in motion control of tobacco guard cells (86), which indicates that pH exhibits features of a bona fide second messenger in plants. Changes in cellular pH are recognized as key cellular regulators, together with Ca^{2+} and ROS, which are involved in diverse physiological processes of plants (31, 141). The resting membrane potential of plant cells is generated by H^+-ATPases, and the slow wave potentials are regulated directly by the *Arabidopsis* H^+-ATPase 1 (AHA1), which was shown to play a role in leaf-to-leaf signaling and plant defense (81).

Optogenetic manipulation of pH in plants could likely be achieved by proton pump rhodopsins and their channel mutants (42, 65, 111) or by high H^+-conductive channelrhodopsins (78, 104, 148). In vivo retinal production paved the way for a successful expression of those rhodopsin variants in plants (161). An optogenetic stimulation of a proton pump might lead to proton efflux while light gating of a proton channel would lead to proton influx. This approach would achieve bidirectional regulation of intracellular pH in a noninvasive manner with precise temporal resolution.

6.3. Lighting up the Black Box of K^+ Homeostasis in Plants

As mentioned above, a light-gated K^+ channel BLINK1 was successfully expressed in *Arabidopsis* to improve plant growth (112). Future applications for in planta use might include channels with faster kinetics and specificity, such as the light-gated K^+ channels SthK-bP (8) or PAC-K (136) with large conductance, which are promising for future plant applications. Recently, a potassium channelrhodopsin *Hc*KCR1 was identified from *Hyphochytrium catenoides* (49). The *Hc*KCR1 could represent an upgrade for K^+ manipulation, assuming successful expression in plant cells.

6.4. Defining the Role of cAMP and cGMP

Optogenetic manipulation of the second messenger cAMP was first carried out in *Drosophila* (131) using the photoactivated adenylate cyclase from *Euglena gracilis* (66). Afterward, a smaller and more

efficient PAC was characterized from the soil bacterium *Beggiatoa* (125, 139). Nowadays, bPAC and its variants (155) or homologs (158) are the most popular optogenetic tools for cAMP manipulation. For the optogenetic manipulation of cGMP, a cyclase opsin (CyclOp) was first identified in the fungus *Blastocladiella emersonii* (6) and then characterized as a light-activated guanylyl cyclase (43, 127, 128). Optogenetic manipulation of cAMP and cGMP in plants would help to dissect their role as second messengers (which is still controversially discussed in plants) in general and to stimulate cyclic nucleotide-gated channels (CNGCs) in planta in particular.

6.5. Allosteric Regulation of Protein Activity and Interactions

Several additional optogenetic applications likely to be introduced into plants include the manipulation of cellular processes by allosteric regulation of protein activity and/or interactions, using LOV-Jα-based tools (154) or light-induced dimer pairs, for example, PHYB/PIF (84, 85, 135, 144), CRY2/CIB1 (73), iLID (54), and TULIPs, in addition to dozens of photoswitches (**https://www.optobase.org/**). Such tools can be implemented for, among other things, the spatiotemporal regulation of protein activity (92, 154), the subcellular targeting of endogenous proteins (44, 157), the induction of phase separation processes (14), protein purification (63, 143), and the subcellular enhancement of protein activities (9, 10). Furthermore, tissue-specific and subcellular application of different tools will help to probe plant responses at a defined cellular or even subcellular level.

7. OPTICAL HARDWARE FOR PLANT OPTOGENETICS

When using rhodopsin- or non-rhodopsin-based tools, two optical parameters are particularly important for optogenetic control of plant processes by light: wavelength and intensity. The spectra of different photosensors/optotools often show an absorption maximum at which wavelength they can best be excited (**Figure 1*b***). If the wavelength for optogenetic stimulation deviates from this, a higher light intensity is needed to produce a comparably strong photoswitch activation. Both laser and LED-based systems can be used as light sources for global or local light applications, with the latter being more frequently used in recent studies. LEDs are inexpensive, have a narrow spectral window, come in a wide range of wavelengths (UV to near-infrared), and can be switched on and off with millisecond time resolution and in a programmable fashion.

To prevent uncontrolled activation of blue/green rhodopsins, plant growth with red light (650- and 660-nm) LED illumination has been set up recently (121, 160, 161). Both commercially available LED arrays and homemade LED systems can be implemented in growth cabinets. The option to install multicolor LED arrays in growth chambers with light intensity control modes as well as timely defined on/off switches is advantageous and a prerequisite for large-scale experimental approaches. In addition, multiwavelength LED panels with programmable spectra, for instance, to simulate defined illumination conditions in terms of spectral quality and intensity or to omit given wavelengths (e.g., green), are currently available from various commercial sources.

In single-plant experiments, however, green light and blue light LEDs can be used for both global and local application of light by means of optical fibers. Direct coupling of LED-based green and blue light into the optics of a widefield microscope was used to trigger stomatal closure of transgenic plants by stimulating *Gt*ACR1 activity in guard cells (64). Making use of green light (530 nm, 17 μW/mm2) LED panels, the transpiration of these transgenic *Gt*ACR1 plants could be light-controlled under background red light conditions (64) that usually fuel stomatal opening and photosynthesis (4). Successful illumination via an optical fiber coupled to a diode-pumped solid state (DPSS) green light (532-nm) laser had already been demonstrated and used to functionally characterize *Gt*ACR1 in tobacco mesophyll cells and pollen tubes (160, 161). However, care must

be taken with light-applications in the minute and hour ranges. A low light intensity is advisable in experiments with long-term illumination to prevent thermal effects through light absorption by the tissue or cells.

Successful local light application via the fluorescence recovery after photobleaching (FRAP) module of a conventional laser scanning microscope (LSM) stimulated *Gt*ACR1 at a small region at the side of the pollen tube apex (161). The use of this methodology is made possible by the rapid light-activation kinetics of *Gt*ACR1, reaching full activity within ~15–20 ms by green light (50, 160, 161), but it requires careful fine-tuning of imaging parameters such as laser power, scan speed, and frame rate. The LSM scan speed within the FRAP experiment was adjusted so that the 514-nm argon laser beam with relatively high laser power (30%) remained on each pixel for ~5 ms, allowing a sufficiently strong *Gt*ACR1 activation (161). At the LSM, the DPSS red light laser (helium–neon 633-nm laser light) can be used for bright-field imaging, since this wavelength does not activate *Gt*ACR1 (see **Figure 1*b***).

For optogenetic activation in small areas or even single cells, lasers can be used, either coupled to or independently from a microscope. Compared to LEDs, lasers are available for a limited number of wavelengths only. However, the advantage of lasers is their high output power and the possibility to focus the laser beam on a local area with high spatial and temporal precision. Alternative technologies for the generation of two-dimensional (2D) and 3D patterns of illumination can be implemented for plant optogenetics. For instance, digital micromirror devices (DMDs) are currently widely employed in bacterial, yeast, and mammalian cell culture to project 2D illumination patterns with up to one million pixels of micrometer-range sizes. Holographic devices are starting to be used to generate 3D illumination patterns in tissues. The range of optical methods used in the field of animal optogenetics far exceeds the range of techniques already used in plant optogenetics (1). Inspired by the multitude of sophisticated optical methods for neuroscience applications, plant optogenetics, which is still in its infancy, will certainly benefit in the future.

8. COMBINING OPTOGENETICS WITH BIOSENSORS

The combination of rhodopsin-based optogenetics with biosensors for the detection of cellular parameters such as cytosolic Ca^{2+} or membrane voltage changes is called all-optical physiology (32, 34). The use of rhodopsins side by side with synthetic genetically encoded biosensors allows monitoring of optotool-driven cellular manipulations and changes of physiology in real time. These all-optical physiology methods represent one of the most informative optogenetic experimental approaches to study biological processes of intact cells. This rapidly expanding field in animal research has enormous potential, but has not yet been implemented in plant science, because rhodopsin-based plant optogenetics has only been established recently (161). Besides aiming to answer physiological questions, the all-optical physiology method is also used in various animal cell systems to verify the functionality of optogenetic tools. For instance, the light dose–dependent increase in cation conductance by ChR2 to induce cytosolic Ca^{2+} increase or membrane depolarization can be determined exactly for any given cellular system. In fact, it is important to determine the functionality of any optotool for each cell system since the expression of rhodopsins and their ability to be targeted to the membrane differ enormously. A major advantage of using biosensors to verify the functionality of optotools is that it is noninvasive, unlike the application of most electrophysiological techniques. Thus, the all-optical approaches monitor the in vivo situation of undamaged or unstressed cells. However, it should be explicitly mentioned that the use of biosensors only adds information for the functional characterization of ion-permeating rhodopsins, while parallel electrophysiological characterizations are still essential. Knowledge of the relative conductivity and activation spectrum as well as activation and deactivation kinetics is of great importance for the interpretation of the physiological responses.

For any optotool, whether stably or heterologously expressed, empirically determining the required light intensity is important to stimulate the rhodopsins efficiently. Using biosensors to visualize the degree of rhodopsin activation is often employed to set up the optimal light conditions for optogenetic experiments. In these experiments, the quantity and quality of light have to be adjusted so that only the necessary amount of light is applied. Otherwise, the simultaneous use of sensors with high light actuator exposure can lead to sensor photobleaching or even to unwanted thermal effects by light absorption.

In addition to the verification of the actuator tools and optimization of light settings for actuator stimulation, the all-optical physiology technique is an optimal way to identify causal relationships between the optogenetically induced stimuli and a physiological response. Cases in which a physiological response can be fine-tuned by the light dose allow conclusions to be drawn about the mechanistic signaling processes in the target tissue.

To perform all-optical physiology experiments, the choice of actuator and sensor pair must be considered carefully. Having the large amount of native plant photoreceptors and the chlorophyll autofluorescence in mind, researchers have proposed various actuator/sensor pairs for possible plant applications (162). As a general rule of thumb, to prevent unwanted activation of the actuator by the sensor imaging approach, one has to consider the excitation spectrum of the sensor to avoid overlap with the excitation spectrum of the actuator in all-optical physiology experiments.

The combination of blue/green light–absorbing optogenetic tools, such as ChR2 and its variants, with red or infrared fluorescent genetic sensors has emerged as a robust technique in animal research to avoid spectral crosstalk (3, 35, 75). The combination of GFP-based sensors with red-absorbing rhodopsins has proven problematic because the red-shifted rhodopsins exhibit significant light absorption in the blue/green spectral range, which can lead to optotool activation during fluorescence sensor excitation (24, 138, 149).

Chlorophyll fluorescence needs to be considered in all-optical physiology experiments in photosynthetic plant tissues, as red autofluorescence from chlorophyll may overlap with sensor fluorescence. However, chlorophyll fluorescence in the range of ~650–750 nm can be successfully excluded by the optical settings combined with using compatible red fluorescent sensors such as the Ca^{2+} sensors R-GECO1 or jRCaMP (28, 159). Advantages or disadvantages of one or the other fluorescent sensor when combined with rhodopsins have been discussed in detail by Zhou et al. (162). However, it is worth mentioning that the Ca^{2+} sensor R-GECO1, the only well-established Ca^{2+} biosensor for plant use (72, 86, 120, 150), may create imaging artifacts during simultaneous optogenetic stimulation with blue and green light (3). High R-GECO1 fluorescence for up to 900 ms was reported to occur after simultaneous optogenetic stimulation with high-power green or blue light, which is caused by the photoswitching effect of R-GECO1 (3). Such problems can be overcome by experimental design, either by using photoswitching-resistant Ca^{2+} sensors such as jRCaMP or by clever imaging design. The photoswitching effect can be circumvented by sequential excitation of R-GECO1 and actuators or other biosensors (86) with a time delay of ~1 s. This is indeed possible in plant cells, because Ca^{2+} increases in plant cells usually last much longer than those of animal cells.

As discussed in Section 4.1, nonopsin-based optogenetic actuators can be combined with genetically encoded quantitative biosensors. This approach allows the interrogation of a signaling or metabolic pathway at the highest spatiotemporal and quantitative resolution (input) and monitors the response at said resolution by using quantitative biosensors targeted to a metabolic proxy of the network (output). A first proof-of-principle experiment was reported for the red-on PHYB optoswitch used to manipulate the levels of an auxin receptor reading out the corresponding output at less-than-nanomolar sensitivity with a ratiometric luminescent auxin biosensor (101). This strategy can be readily extrapolated to other phytohormones or signaling networks.

9. IS THERE A FUTURE IN FIELD APPLICATION?

Translational application of optogenetics is already highly anticipated in the therapeutic field, especially for vision restoration (126). Plant optogenetics is at its early stage, where it is improving basic research. Since crop farming takes place under the sun, field application of plant optogenetics seems complex. However, there are several aspects that could bring plant optogenetics into field application.

The red light–activated, blue light–repressed optogenetic tool PULSE (106) is useful to induce the expression of different optogenetic tools at a certain development or physiological stage or environmental condition. For example, upon stress hardships, the plant can be primed upon optogenetic stimulation for enhanced tolerance, or alternatively, a given developmental or plant life fate can be triggered on demand. Additionally, tissue-specific promoters can be used to restrict the expression timing and area in the plant. Furthermore, less sensitive optogenetic tools that can only be activated at high light intensities will enable the growth of plants in greenhouses or fields under mild white light conditions, where the optogenetic systems will be off, and they may be activated by using high-intensity light of the specific inducing wavelengths. In addition to this, a combination of less sensitive photoreceptors with tissue-specific expression could also facilitate field application. For example, expression of the less sensitive ZipACR (52, 160) in the stomata might help increase stomatal closure under high sunlight conditions to reduce transpiration. We also hypothesize that light-gated ion H^+, Cl^-, and Na^+ pumps (162) could help to build the proton and electric gradients or improve sodium exclusion, which might facilitate plant growth and thus a future field application.

SUMMARY POINTS

1. Optogenetics is extremely successful in neuroscience but is now expanding to further animal research areas. Its application in plant biology has to tackle the requirement of plants to grow in light and the need for nonactivating illumination. Several approaches have recently achieved such growth conditions.
2. The long-sought goal of overcoming the problem of inadvertent activation of optogenetic tools in plants under normal growth conditions, namely cycles of darkness/white light, was achieved by multiplexing two different optoswitches. A red-on and a blue-off gene expression switch were combined to engineer an optogenetic system that is off under sunlight and in the dark and is activated only upon irradiation with monochromatic red light, enabling the use of the plant-usable light-switch element (PULSE) system in whole plants.
3. As microbial rhodopsins are still the most powerful optogenetic tools and many of them are not activated by red light, growth of model plants under red light-emitting diode (650-nm) illumination was tested, yielding healthy plants in which the expressed rhodopsin was silent until illuminated with green light.
4. Rhodopsins need the essential cofactor retinal (vitamin A aldehyde) to absorb light and become functional. Land plants contain no endogenous rhodopsins and no retinal, but their chloroplasts are full of β-carotene (provitamin A). By introducing a β-carotene-splitting enzyme (dioxygenase) into the chloroplasts, we generated healthy plants with enough retinal to support functional rhodopsin expression.

5. Rhodopsins allow noninvasive disturbance of membrane potential and cytosolic pH or Ca^{2+}. The successful generation of tobacco plants with functional Anion Channelrhodopsin-1 allowed for noninvasive probing of plant cell fluid loss, stomata closing, and pollen tube growth. Further applications will follow with the expression of H^+- or Ca^{2+}-permeable rhodopsins.
6. A first generation of optogenetic switches for the control of nuclear and plastid gene expression inducible with wavelengths ranging from blue to green to red/far-red light have been engineered and applied in plant cells and whole plants.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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