New Phytologist

Forum

Viewpoints

The green light gap: a window of opportunity for optogenetic control of stomatal movement

Summary

Green plants are equipped with photoreceptors that are capable of sensing radiation in the ultraviolet-to-blue and the red-to-far-red parts of the light spectrum. However, plant cells are not particularly sensitive to green light (GL), and light which lies within this part of the spectrum does not efficiently trigger the opening of stomatal pores. Here, we discuss the current knowledge of stomatal responses to light, which are either provoked via photosynthetically active radiation or by specific blue light (BL) signaling pathways. The limited impact of GL on stomatal movements provides a unique option to use this light quality to control optogenetic tools. Recently, several of these tools have been optimized for use in plant biological research, either to control gene expression, or to provoke ion fluxes. Initial studies with the BL-activated potassium channel BLINK1 showed that this tool can speed up stomatal movements. Moreover, the GL-sensitive anion channel *Gt*ACR1 can induce stomatal closure, even at conditions that provoke stomatal opening in wild-type plants. Given that crop plants in controlled-environment agriculture and horticulture are often cultivated with artificial light sources (i.e. a combination of blue and red light from light-emitting diodes), GL signals can be used as a remote-control signal that controls stomatal transpiration and water consumption.

Introduction

Optogenetics offers the possibility of controlling physiological processes in a minimally invasive way, with the use of demand-adapted light protocols (Christie & Zurbriggen, 2021; Emiliani *et al.*, 2022). This approach has revolutionized neurobiology, since light-activated ion channels can be expressed in specific neurons and used to control the excitability of these cells (Rost *et al.*, 2017). More recently, optogenetic tools have also been developed for plants cells, to control the expression of key genes (Muller *et al.*, 2014; Ochoa-Fernandez *et al.*, 2020; Christie & Zurbriggen, 2021), manipulate the plasma membrane potential (Reyer *et al.*, 2020; Zhou *et al.*, 2021b), and direct the movement of guard cells, which open and close stomata (Papanatsiou *et al.*, 2019; Huang *et al.*, 2021).

Unlike most mammalian cells, plant cells use an array of native photoreceptors to adapt their physiology and morphogenesis in response to diurnal and seasonal changes in ambient light conditions (Fig. 1a) (Kami *et al.*, 2010). Plant cells possess photoreceptors that are activated by red light (RL), blue light (BL) and ultraviolet light, but so far no receptor has been identified that is specifically stimulated by green light (GL). Green light does affect photomorphogenesis, since it reverses the BL-dependent activation of cryptochromes (Kami *et al.*, 2010; Smith *et al.*, 2017), but these responses are moderate compared to those triggered by RL and BL. Likewise, GL can drive photosynthesis (Terashima *et al.*, 2009), but chloroplasts absorb GL with only a low efficiency (Fig. 1a). We therefore propose that the GL gap in photoreception may provide a window in the light spectrum that can be used to activate optogenetic proteins (Fig. 1b) while having a minimal impact on the physiology and morphology of plants (off-target effects).

One can ask: 'Why are plants virtually blind to the green part of the solar spectrum?'. The answer may be found in the evolution of oxygenic photosynthesis, which most likely emerged in cyanobacteria. Modern relatives of these bacteria possess rhodopsin-based ion pumps (Hasegawa *et al.*, 2020), which enable them to convert light-energy into ion gradients. It is likely that ancient cyanobacteria already possessed rhodopsins, which transported ions with the aid of GL. Chlorophyll may have evolved as an additional light-harvesting pigment for photosynthesis in these organisms, with an absorption-spectrum that did not interfere with that of the type I rhodopsins (Mirkovic *et al.*, 2017). As a result, these ancient bacteria could use the full spectrum of visible light – BL and RL were absorbed by Chl, while the bacterio-rhodopsins mainly captured GL (Rein & Deussing, 2012). Later in the evolutionary process, the rhodopsins were lost in land plants and left a gap in the absorption spectrum that has turned our planet green. Today we can make use of these properties that developed early in evolution and exploit the GL gap of chloroplasts to stimulate plant cells with GL-dependent optogenetic tools.

Endogenous responses of guard cells to RL and BL

There are two well-established stomatal opening responses to light: one is induced by photosynthetically active radiation (PAR), while the other is a photosynthesis-independent response that is induced by BL. The PAR response of stomata is often called the RL-response, but this can be misleading, as it is driven both by BL and RL. By contrast, the BL-specific response is not sensitive to RL, since it is triggered by the BL-absorbing phototropin receptors (Fig. 1a) (Kinoshita *et al.*, 2001; Christie, 2007).

Photosynthetically active radiation-induced stomatal opening

Hypotheses regarding the response of stomata to PAR were formulated as early as the 1960s, and were based on the spectral

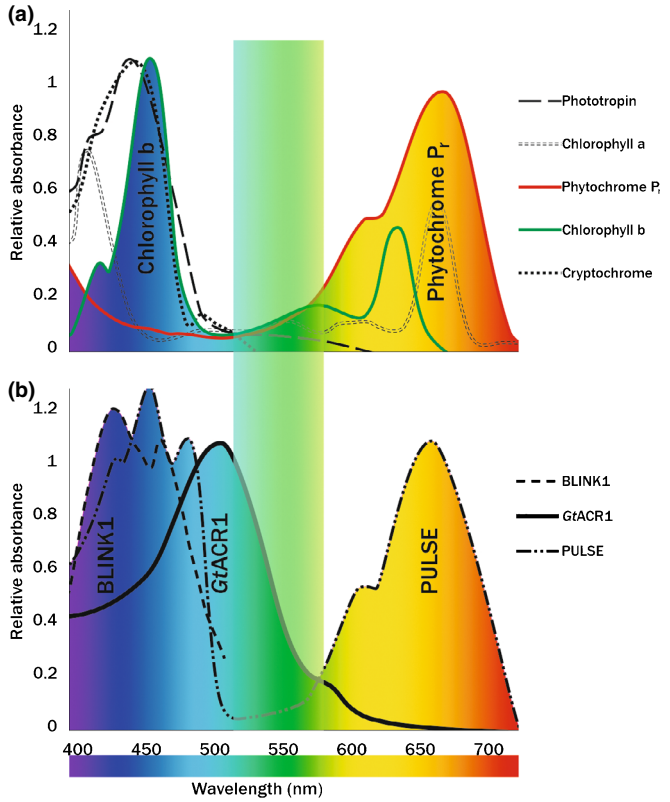

Fig. 1 Action, or absorption, spectra of photoreceptors and optogenetic proteins. (a) Absorption spectra of Chla and *b*, the blue light (BL)-sensing phototropins and cryptochromes, and the red light (RL)-absorbing phytochromes. Note the green light (GL) gap in the absorption spectrum (indicated by the green bar), which can be used to activate the anion channelrhodopsin 1 in *Guillardia theta* (*Gt*ACR1, Wietek *et al*., 2016). (b) Comparison of the action spectrum of *Gt*ACR1 with the absorption of BL-induced K^+ channel 1 (BLINK1, Cosentino *et al*., 2015) and plant usable light-switch elements (PULSE, spectra derived from PhyB, Golonka *et al*., 2019; and EL222, Nash *et al*., 2011).

dependency of stomatal opening. It turned out that the opening of stomata was efficiently induced by BL and RL, but stomata were less sensitive to light in the green-to-yellow part of the spectrum (Kuiper, 1964). This spectral dependency thus mirrored that of Chl's, and in line with the important role of Chl, PAR-induced stomatal opening is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DMCU), a specific blocker of linear electron transport in the thylakoid membrane (Kuiper, 1964; Schwartz & Zeiger, 1984).

Ion uptake in guard cells depends on active plasma membrane H^+-ATPases (Kollist *et al*., 2014; Inoue & Kinoshita, 2017) and studies with intact Arabidopsis leaves showed that PAR stimulates phosphorylation of the C-terminal domain of H^+-ATPases (Ando & Kinoshita, 2018), which activates these H^+ pumps (Shimazaki *et al*., 2007). It has been suggested that PAR also promotes the production, by guard cell chloroplasts, of ATP, which is the substrate of H^+-ATPases (Tominaga *et al*., 2001). However, recent data obtained using a genetically encoded ATP sensor showed that guard cell chloroplasts of Arabidopsis have only a very limited ATP production capacity. In fact, these chloroplasts take up ATP from the cytosol in the light to support starch synthesis (Lim *et al*., 2022). It is thus likely that PAR provokes stomatal opening via another signal, such as the intracellular ATP level in guard cells.

Several studies have suggested a strong link between CO_2 sensing and stomatal PAR responses. Suppression of MAP kinase 4 (MPK4) in tobacco (Marten *et al*., 2008) and loss of MPK4 and MPK12 in Arabidopsis (Des Marais *et al*., 2014; Jakobson *et al*., 2016; Tõldsepp *et al*., 2018) has been shown to result in plants that do not close their stomata under high atmospheric CO_2 concentrations or in darkness. A similar result was obtained with mutants that had either lost the MAP triple kinase high temperature1 (HT1), which acts downstream of MPK12 (Fig. 2; Jakobson *et al*., 2016), or carbonic anhydrases1 and 4 (CA1 and 4), which are likely to function upstream of MPK4 and MPK12 (Hu *et al*., 2010). Loss-of-function mutants of the latter gene families were also impaired in stomatal opening induced by PAR and low atmospheric CO_2 concentrations (Hashimoto *et al*., 2006; Hu *et al*., 2010; Matrosova *et al*., 2015). This strongly suggests that PAR induces a reduction in CO_2 concentrations in intact leaves, as well as the guard cells therein, which triggers stomatal opening (Roelfsema *et al*., 2002; Roelfsema & Hedrich, 2005). In other words, guard cells monitor the status of mesophyll carbon assimilation and respond to low internal CO_2 concentrations by opening stomatal pores.

In line with the commonalities between PAR and low CO_2 responses, both stimuli inhibit the activity of S-type anion channels in guard cells (Brearley *et al*., 1997; Roelfsema *et al*., 2002; Marten *et al*., 2008; Hiyama *et al*., 2017). These channels are encoded by slow anion channel 1 (SLAC1) and SLAC1-homolog 3 (SLAH3) in Arabidopsis and enable the efflux of anions (Negi *et al*., 2008; Vahisalu *et al*., 2008; Geiger *et al*., 2010; Hedrich & Geiger, 2017). The activation of the guard cell's endogenous SLAC1- and SLAH3-type anion channels will depolarize guard cells and provoke stomatal closure, as discussed for the optogenetic tool *Gt*ACR1 (see The current state of optogenetic control of stomatal movements section). PAR will lower the CO_2 concentration in leaves and thus inhibit S-type anion channels, which will prevent the efflux of anions and allow hyperpolarization of the guard cell plasma membrane (Roelfsema *et al*., 2002; Marten *et al*., 2008). As a result, the inhibition of S-type anion channels by PAR will counteract stomatal closure and instead stimulate stomatal opening.

Blue-light induced stomatal opening

Experiments in the 1980s showed that the stomata in *Xanthium strumarium* have a higher sensitivity to BL than RL (Sharkey & Raschke, 1981) and that the stomata in *Commelina communis* responded to BL irradiation even if their photosynthesis was saturated with RL (Iino *et al*., 1985). It turned out that these BL-specific responses depend on the phototropins 1 and 2 (PHOT1 and 2) in Arabidopsis (Fig. 2, Kinoshita *et al*., 2001). PHOT1 and 2 are protein kinases whose activity is repressed in darkness but activated in the presence of BL (Christie, 2007; Shimazaki *et al*., 2007). The changes in ion transport evoked by PHOTs are similar to those resulting from PAR, since they stimulate the activity of plasma membrane H^+-ATPases (Kinoshita &

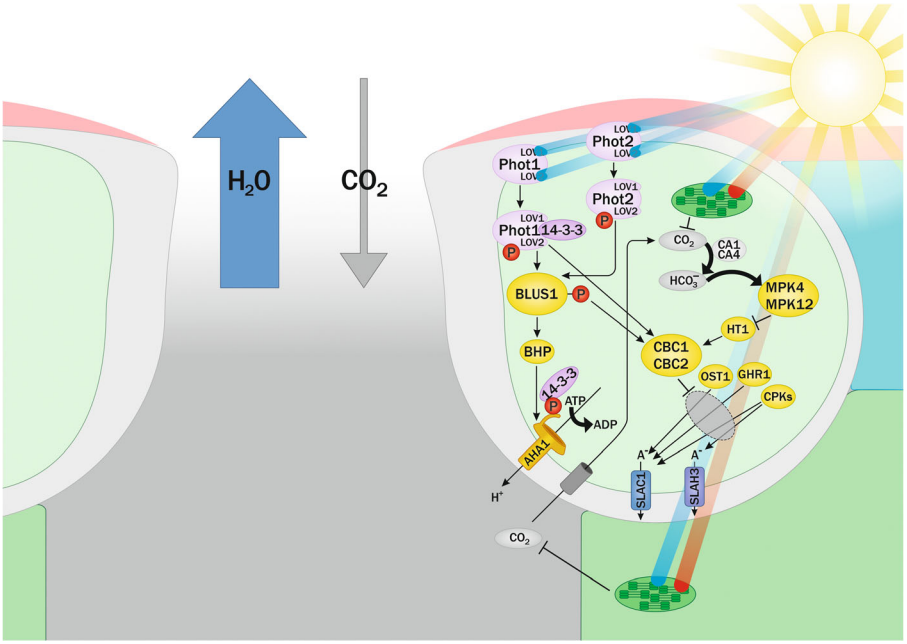

Fig. 2 Illustration of light signaling pathways that provoke stomatal opening in guard cells. In Arabidopsis, light acts via a blue light (BL)-specific signaling pathway that is initiated by phototropin1 and 2 (Phot1/Phot2), which possess two BL-sensing light oxygen voltage (LOV) domains. In addition, a photosynthetically active radiation (PAR, or red light) branch is present, which depends on chloroplast activity. The phototropins, bind 14-3-3 proteins and activate the plasma membrane H^+-ATPases (mainly AHA1) via BL signaling 1 (BLUS1) and BL-dependent H^+-ATPase phosphorylation (BHP) (indicated by arrows) and inhibit the slow anion channel 1 (SLAC1) and SLAC1-homolog 3 (SLAH3) via Converge of BL and CO_2 1 and 2 (CBC1 and 2, inhibition is indicated by blunt-ended arrows). CBC1 and 2 are likely to interfere with the mechanism by which three protein kinases, open stomata 1 (OST1), guard cell hydrogen peroxide-resistant 1 (GHR1) and the calcium-dependent protein kinases (CPKs), stimulate the activity of SLAC1 and SLAH3. Photosynthesis in mesophyll cells and the guard cell itself lowers the cytosolic CO_2 concentration in guard cells, which will also result in a lower hydrogen carbonate (HCO_3^-) concentration (CO_2 is converted into HCO_3^- by carbonic anhydrase 1 and 4 (CA1 and CA4)). The drop in HCO_3^- concentration is likely to reduce the activity of MAP-kinase 4 and 12 (MPK4 and 12), which inhibits high temperature 1 (HT1). As a result, HT1 is activated and stimulates the activity of CBC1- and 2, which in turn represses the mechanism by which OST1, GHR1 and CPKs stimulate the activity of SLAH3 and SLAC1, as explained for BL signaling.

Shimazaki, 1999; Kinoshita *et al.*, 2001) and inhibit S-type anion channels (Marten *et al.*, 2007; Hiyama *et al.*, 2017). Both the PAR and BL signal pathways thus seem to act in parallel and target the same transporters in the plasma membrane of guard cells (Fig. 2).

In line with the similarities in the PAR (BL- and RL-driven) and BL-specific responses, their signaling pathways were found to join at two MAP triple kinases, which were named Converge of BL and CO_2 1 and 2 (CBC1 and 2). Loss of CBC1 and 2 caused impaired responses to PAR and BL and the inability of both stimuli to regulate S-type anion channels (Hiyama *et al.*, 2017). However, upstream of CBC1/2, the PAR and BL signaling pathways are likely to differ, since PAR probably regulates CBC1/2 via interaction with HT1 (Hiyama *et al.*, 2017), which is important in PAR and CO_2 signaling, but not for BL responses (Hashimoto *et al.*, 2006; Matrosova *et al.*, 2015).

By contrast, BL controls the activity of CBC1 via interaction with the Ser/His protein kinase blue light signaling 1 (BLUS1) (Takemiya *et al.*, 2013; Hosotani *et al.*, 2021), which leads to inhibition of the S-type anion channels (Marten *et al.*, 2007; Hiyama *et al.*, 2017). In addition, the BL pathway has a second branch that causes activation of H^+-ATPases, which involves an interaction between BLUS1 and the Raf-like protein kinase known as BL-dependent H^+-ATPase phosphorylation (BHP) (Hayashi *et al.*, 2017). Phototropins thus seem to stimulate the activity of H^+-ATPases via BHP, while that of anion channels is inhibited via CBC1 and 2.

It should be noted that this role for CBC1 and 2 was challenged in a recent study by Hayashi *et al.* (2020), who showed that CBC1 and 2 also interact with PHOT1/2 and regulate H^+-ATPases. They concluded that CBC1 and 2 have a negative impact on stomatal opening, in contrast to the findings of Hiyama *et al.* (2017), who reported that both protein kinases stimulate stomatal opening. For simplicity, we will focus on the regulation of S-type anion channels by CBC1/2 and, since the disagreement regarding the impact of these protein kinases on stomata has not yet been resolved, we will assume that they stimulate stomatal opening.

The BL-specific response of stomata probably developed early in evolution, since it is found in a wide range of vascular plants, from lycophytes to seed plants (Doi *et al.*, 2015; Sussmilch *et al.*, 2019). Nevertheless, a recent survey among various seed plants revealed a high degree of variation in the BL-responsiveness of stomata (Vialet-Chabrand *et al.*, 2021). While the stomata of Arabidopsis and rice displayed strong BL-specific responses, their counterparts in potato and tobacco showed virtually no difference in their responses to BL and RL. Apparently, the stomata of several crop plants have lost their BL-specific response, just as previously found for the clade of *Polypodiopsida* ferns (Doi *et al.*, 2015). The following question therefore arises: why do the stomata of most plant species display the photosynthesis-independent BL-response, in addition to the common PAR (BL and RL) response, while in some others it has been lost?

The current state of optogenetic control of stomatal movements

A well-equipped toolbox of light-gated channels and pumps has been established for neurobiology, and several of these tools belong to the type I rhodopsins (Rost *et al.*, 2017). This group of proteins includes the Channelrhodopsins (ChRs), which act as light-gated ion channels that gain their light-sensitivity from the chromophore 'all-*trans* retinal'. The use of these tools also enables the manipulation, in terms of both space and time, of ion transport in plant tissues using tailored light beams and specific protocols. For instance, the expression of the cation channel Channelrhodopsin 2 (ChR2) in Arabidopsis and tobacco was used to trigger changes in the membrane potential of mesophyll cells via light pulses (Reyer *et al.*, 2020). In this pioneering study, the activity of ChR2 was still dependent on the external feeding of synthetic retinal (Reyer *et al.*, 2020). In a follow-up project, the bacterial β-carotene dioxygenase (*Mb*Dio) was transplanted into chloroplasts of tobacco, which enabled these plants to produce their own retinal (Zhou *et al.*, 2021b). In addition, the light-activated anion channelrhodopsin of the algae *Guillardia theta* (*Gt*ACR1) was expressed, and as a result, cells could be depolarized with GL pulses in intact tobacco plants (Zhou *et al.*, 2021b).

Tobacco plants that simultaneously expressed *Mb*Dio and *Gt*ACR1 were also used to resolve the long-standing question of whether the opening of plasma membrane anion channels in guard cells is sufficient to close stomata. It turned out that activation of the *Gt*ACR1 anion channel provoked an efflux of anions and membrane depolarization in guard cells (Fig. 3; Huang *et al.*, 2021). Due to this depolarization, voltage-dependent K^+ efflux channels of the guard cell outward rectifying K^+ channel-type (GORK-type) became activated (Ache *et al.*, 2000; Hosy *et al.*, 2003), leading to a simultaneous release of anions and K^+. As a result, the osmotic content of the motor cells decreased and provoked stomatal closure (Fig. 3; Raschke *et al.*, 1988; Roelfsema & Hedrich, 2005; Kim *et al.*, 2010).

A ChR-independent optogenetics approach to modulating stomatal movement was taken, using the BL-activated K^+ channel BLINK1 (Papanatsiou *et al.*, 2019). BLINK1 is based on the viral K_{CV} channel, which is coupled to the BL sensing domain of oat phototropin 1 (Cosentino *et al.*, 2015). Activation of BLINK1 in Arabidopsis guard cells enhanced the velocity at which stomata opened and closed, particularly under growth conditions where light levels fluctuated rapidly (Papanatsiou *et al.*, 2019). BLINK1 thus stimulated stomatal movement but did not affect the direction in which the movement occurred (opening vs closure). In contrast to BLINK1, activation of the *Gt*ACR1 was a game changer. Stimulation of the *Gt*ACR1 anion channel caused stomatal closure, even at conditions that provoked stomatal opening in control (non *Gt*ACR1 transformed) plants (Fig. 3; Huang *et al.*, 2021).

Potential use of optogenetic tools in agriculture and horticulture

If plants are grown under RL only, the light-gated anion channel *Gt*ACR1 can be expressed with no impact on the growth rate (Zhou

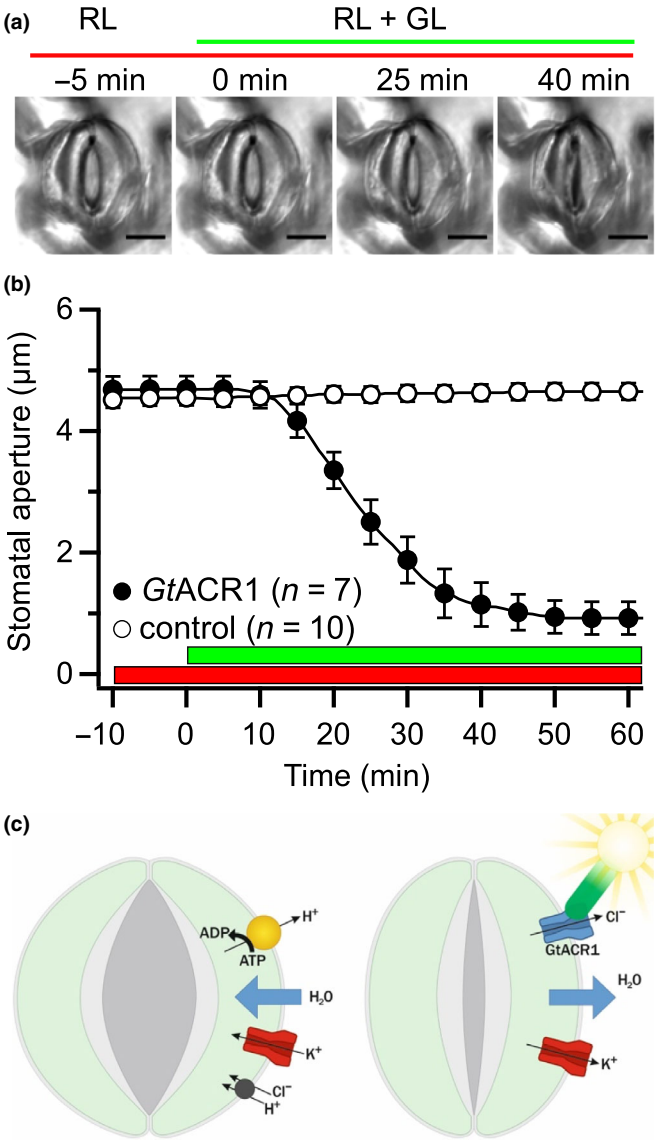

Fig. 3 Green light (GL) provokes stomatal closure in tobacco plants that express anion channelrhodopsin 1 of *Guillardia theta* (*Gt*ACR1). (a) Stoma in an intact tobacco leaf, in which the light-gated anion channel *Gt*ACR1 is expressed (Huang *et al.*, 2021). Stomatal opening was provoked using red light (RL) (λ = 630 nm, 0.018 mW mm^{-2}) and images were obtained before, at the time of, and at two time points after commencing stimulation with GL (λ = 525 nm, 0.57 mW mm^{-2}), as indicated by the colored bars above the images. Bars, 15 µm. (b) Average data for GL-induced responses of stomata that express *Gt*ACR1 (closed circles, $n = 7$) and controls (open circles, $n = 10$). The red bar indicates stimulation with photosynthetically active radation (PAR) (λ = 630 nm, 0.018 mW mm^{-2}), while the green bar shows the period during which stomata were stimulated with GL (λ = 525 nm, 0.57 mW mm^{-2}). Error bars represent SE; data are from Huang *et al.* (2021). (c) Illustration depicting how stomatal opening under RL is driven by the H^+-ATPase, which enables the uptake of potassium (K^+) via K^+ channel of *Arbidopsis thaliana* 1-like (KAT1-like) ion channels and uptake of chloride (Cl^-) via a Cl^-/H^+ co-transporter (left image). The accumulation of K^+ and Cl^- drives the uptake of H_2O and the swelling of guard cells. Activation of *Gt*ACR1 with GL provokes the efflux of Cl^- and depolarizes the guard cells, which causes the simultaneous efflux of K^+ via guard cell outward rectifying K^+ channel-like (GORK-like) K^+ channels (right image). Due to the loss of osmolytes, the guard cells shrink and the stomatal pore closes.

et al., 2021b). This observation agrees with the finding that RL is incapable of activating *Gt*ACR1 (Fig. 3; Govorunova *et al.*, 2015; Wietek *et al.*, 2016; Zhou *et al.*, 2021b). However, the application of BL is more challenging, as ChRs tend to be activated to some degree by BL, even if their peak activity occurs only in the GL part of the spectrum (Prigge *et al.*, 2012; Wietek *et al.*, 2016). A future goal, therefore, will be to generate ChR variants that are less sensitive to BL, so that BL can be applied at an intensity high enough to optimize plant growth and low enough not to activate the optogenetic tools.

Type I rhodopsins can display a wide range of spectral properties, depending on the species from which they were obtained. For instance, the action spectrum of VChR1 of *Volvox carteri* is shifted, by *c.* 80 nm, to a longer wavelength than that of ChR2 from *Chlamydomonas reinhardtii* (Zhang *et al.*, 2008). Based on the differences between the ChRs of both species and a targeted mutagenesis approach, ChR2 could be color-tuned to channels that have peak absorption values ranging from 460 to 560 nm (Prigge *et al.*, 2012). Similar differences in spectral properties have also been described for light-gated anion channels (Wietek *et al.*, 2016; Zhou *et al.*, 2021a). So far, it seems that the use of *Gt*ACR1 is optimal for use in future studies, since it has an action spectrum with a peak value at 518 nm that nicely complements the green gap of Chls (Fig. 2; Wietek *et al.*, 2016). Alternatively, one may use MerMAID light-activated anion channels, which were discovered recently (Oppermann *et al.*, 2019). MerMAIDs rapidly inactivate during prolonged illumination, which makes it feasible to design light protocols that activate these anion channels on demand, while they occur in an inactivated state during continuous BL illumination.

In controlled-environment agriculture and horticulture, plants are often grown with LED illumination systems that provide only BL and RL (Bantis *et al.*, 2018). The addition of GL does influence plant growth (Wang & Folta, 2013; Smith *et al.*, 2017), but this light quality is not essential for the growth of many crops. It is thus feasible to grow plants with BL and RL, while using GL to activate rhodopsin-based optogenetic tools, such as *Gt*ACR1. Plants that express *Gt*ACR1 in guard cells can be used to close the stomata of selected leaves upon demand. This approach may be useful to redirect sap flows or prevent the infestation of crop plants by pathogenic microorganisms, as explained below.

GL-induced closure of stomata can be used to prevent Ca^{2+} deficiency in fast growing plant tissues. Diseases due to Ca^{2+} deficiency are known as 'tip burn' in young expanding leaves, 'black heart' in enclosed tissues like celery and 'blossom end rot' in fruits such as tomato (White & Broadley, 2003). These fast-growing tissues are predominantly fed by the phloem but will obtain Ca^{2+} mainly via the xylem sap (White & Broadley, 2003). As a result, the Ca^{2+} supply will be limited and will be reduced further if the xylem sap pressure drops, due to high transpiration rates in mature leaves. In line with this dependency, blossom end rot of tomato fruits could be prevented via an abscisic acid (ABA)-induced reduction in transpiration in mature leaves (de Freitas *et al.*, 2014). In the future, optogenetic tools, such as *Gt*ACR1, could also be used to lower the transpiration of mature leaves and thereby prevent Ca^{2+} deficiency in phloem-fed tissues. Even though this approach will also reduce photosynthesis, it is likely that the prevention of Ca^{2+} deficiency will outweigh the penalty of a temporary reduced growth rate.

In addition, *Gt*ACR1 may be used to prevent infection by pathogenic microorganisms that use open stomata to enter their host. Upon recognition of such microorganisms by guard cells, the stomata will close and thereby prevent a further infestation (Melotto *et al.*, 2006). However, some virulent pathogens have evolved means of counteracting this defense response and triggering the re-opening of stomata (Melotto *et al.*, 2006; McLachlan *et al.*, 2014). Here, optogenetics may provide a solution, since stomata can be closed with *Gt*ACR1, independently of native signal transduction pathways in guard cells. The GL-induced activation of *Gt*ACR1 can thus overrule the reopening response evoked by virulent microbes and stop further infestation.

Outlook

Various new optogenetic tools are likely to become available for plant research soon, which may help provide answers to major long-standing questions concerning control of stomatal movements. For instance, cytosolic Ca^{2+} and pH signals have been implicated in the signaling pathways of guard cells (Siegel *et al.*, 2009; Huang *et al.*, 2019; Li *et al.*, 2021). Several ChRs have been developed that conduct H^+ and Ca^{2+} (Rost *et al.*, 2017), and these tools are predestined to provide new insights into the guard cell signaling network. Moreover, light-gated ChR-type ion channels can also be targeted to intracellular compartments, such as the vacuole and endoplasmic reticulum (ER), to attempt to answer questions regarding the role of these organelles in Ca^{2+}-dependent signaling pathways in guard cells.

A second promising approach is the light-controlled expression of genes, with a specific role in guard cells. The plant usable light-switch elements (PULSE) system developed by Ochoa-Fernandez *et al.* (2020) allows the onset of gene expression at illumination with RL only, whereas the expression can be switched off by the additional application of blue, white, or infrared light. This approach will be useful in studying the roles of specific members of multigene families that have been proposed to play important roles in the regulation of stomatal movement, such as the Ca^{2+}-dependent protein kinases (CPKs) (Geiger *et al.*, 2010; Roelfsema *et al.*, 2012; Schulze *et al.*, 2021), CBL-interacting protein kinases (CIPKs) (Cheong *et al.*, 2007; Inoue *et al.*, 2020), or MAP-triple protein kinases (Lin *et al.*, 2020; Takahashi *et al.*, 2020). Members of these gene families can be cloned into the PULSE system and will not be expressed, as long as the plants are grown under white light. In RL only, the gene of interest will be expressed and the impact of the encoded protein on stomatal movements can be studied in detail. So far, the PULSE system has been used to induce a global expression of specific genes, but it may be possible to confine expression to guard cells with the use of guard-cell specific promotors.

The PULSE system may eventually be exploited to prevent the unintended responses evoked by optogenetic tools during plant development. For instance, the expression of light-gated channels can be repressed by PULSE under white light, while the channel gene of interest will be transcribed under RL. As soon as the channel

gets expressed under RL, GL pulses can be used to provoke the opening of the channel and study its impact on the control of stomatal movements. The combined use of several optogenetic tools is therefore likely to have a bright future in plant biology.

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