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Perspective

Exciting new discoveries in phytochrome-mediated light signaling pathways

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How organisms perceive and respond to their surrounding environment is a fundamental question in biology. This is particularly relevant to plants because of their sessile nature. Light and temperature are two environmental factors that have profound effects on plant growth and development. Plants have evolved intricate regulatory modules to translate these environmental signals into their developmental programs, from germination to flowering [1]. Phytochromes (phys) are one such red/far-red light sensory photoreceptors ubiquitously present in organisms ranging from bacteria to plants [2]. Phytochromes exist in the red light absorbing (Pr) form in darkness and are converted to a biologically active far-red light absorbing (Pfr) form upon exposure to red light. The Pfr form can be converted back to Pr form upon far-red light exposure, thus phytochromes are acting like light switches that can be turned "on" and "off". Because phytochromes were known to be in the cytosol in a plant cell, a major focus in the phytochrome signaling field in the mid-70s to mid-90s was to find second messengers that mediate the light signal from the cytoplasm to the nucleus. A number of research articles showed the involvement of calcium (Ca^{2+}), calmodulin and cGMP in mediating light signaling to control gene expression and chloroplast development [3]. However, the focus quickly shifted and remained on to nucleus when a milestone study showed that phytochromes translocate from cytosol to the nucleus in response to light [4]. After more than two decades of intense focus on the nuclear phytochrome signaling mechanisms, Zhao et al. [5] circled back to the importance of cytosolic Ca^{2+} in mediating phytochrome signaling not as a second messenger but as a regulator of phytochrome nuclear translocation.

Ca^{2+} not only acts as a mineral, but also is a versatile regulator of a complex and sophisticated network of signaling pathways, regulating plant growth, development, and stress responses [6,7]. In plants, Ca^{2+} signals are mediated by changes in free cytosolic Ca^{2+}, [Ca^{2+}]cyt. Different stimulus-induced [Ca^{2+}]cyt include specific periods and amplitude, which could be perceived and decoded by Ca^{2+}-binding proteins, containing Ca^{2+} binding domains, like calmodulin. Calcium signaling is involved in phototransduction [7]. Red and blue light cause [Ca^{2+}]cyt increase and Ca^{2+} promotes light-responsive gene expression [7]. However, the underlying mechanism is unclear. Zhao et al. [5] filled this gap and bridge the Ca^{2+} signaling and phytochrome signaling pathways by characterizing a phytochrome B (phyB)-Ca^{2+}-CPK6/12-phyB loop to promote phyB translocation into the nucleus in response to light signals (Fig. 1). They show that red light induces a robust [Ca^{2+}]cyt increase in etiolated *Arabidopsis* seedlings, which is phyB-dependent. The increased [Ca^{2+}]cyt activates two calcium-dependent protein kinases, CPK6 and CPK12, which promote phyB phosphorylation at S80 and S106. S80/S106 phosphorylation is required for phyB nuclear translocation to initiate light responses.

Calcium and light signaling. Zhao et al. [5] demonstrated short red light exposure (30 s) induces a transient increase in [Ca^{2+}]cyt, followed by a rapid decay to basal level within 2 min. However, red light fails to induce [Ca^{2+}]cyt increase in *phyB-9* mutant background and [Ca^{2+}]cyt induction by red light is reversible by far-red light, suggesting that phyB mainly mediates this [Ca^{2+}]cyt induction in response to red light. Similarly, blue light can also induce [Ca^{2+}]cyt increase relying on the blue light photoreceptor phototropin, rather than cryptochromes [8]. Blue light exposure triggers Ca^{2+} influx from the apoplast via the phototropin-activated Ca^{2+}-permeable channel on the plasma membrane. It is possible that blue light activates phototropins and then phototropins with other components activate the Ca^{2+}-permeable channel, causing Ca^{2+} influx. A similar case might be involved in phyB-dependent [Ca^{2+}]cyt increase in the cytosol. However, the identity of the Ca^{2+}-permeable channel mediating this process under red light is still unknown. Different light spectrums activating specific receptors to activate the Ca^{2+} channel to induce [Ca^{2+}]cyt increase might be an important strategy for plants to distinguish the initial input signals. However, it is equally interesting to understand how plant decodes the same [Ca^{2+}]cyt increase to regulate specific output.

phyB phosphorylation and nuclear translocation. All phytochromes have been shown to translocate into the nucleus [9]. However, the mechanism of only phyA nuclear import has been well-understood. Photoactivated phyA interacts with two plant-specific proteins called far-red elongated hypocotyl 1 (FHY1) and FHY1-like (FHL), both of which have nuclear location signal (NLS). FHY1/FHL-phyA complex is then imported by the well-known importin complexes into the nucleus [9]. However, the mechanism of nuclear import of other phytochromes including phyB is less understood. It has been proposed that phyB has a cryptic NLS present within the C-terminal PAS-related domain, which is masked in the Pr form, and the light-induced Pfr photoconversion

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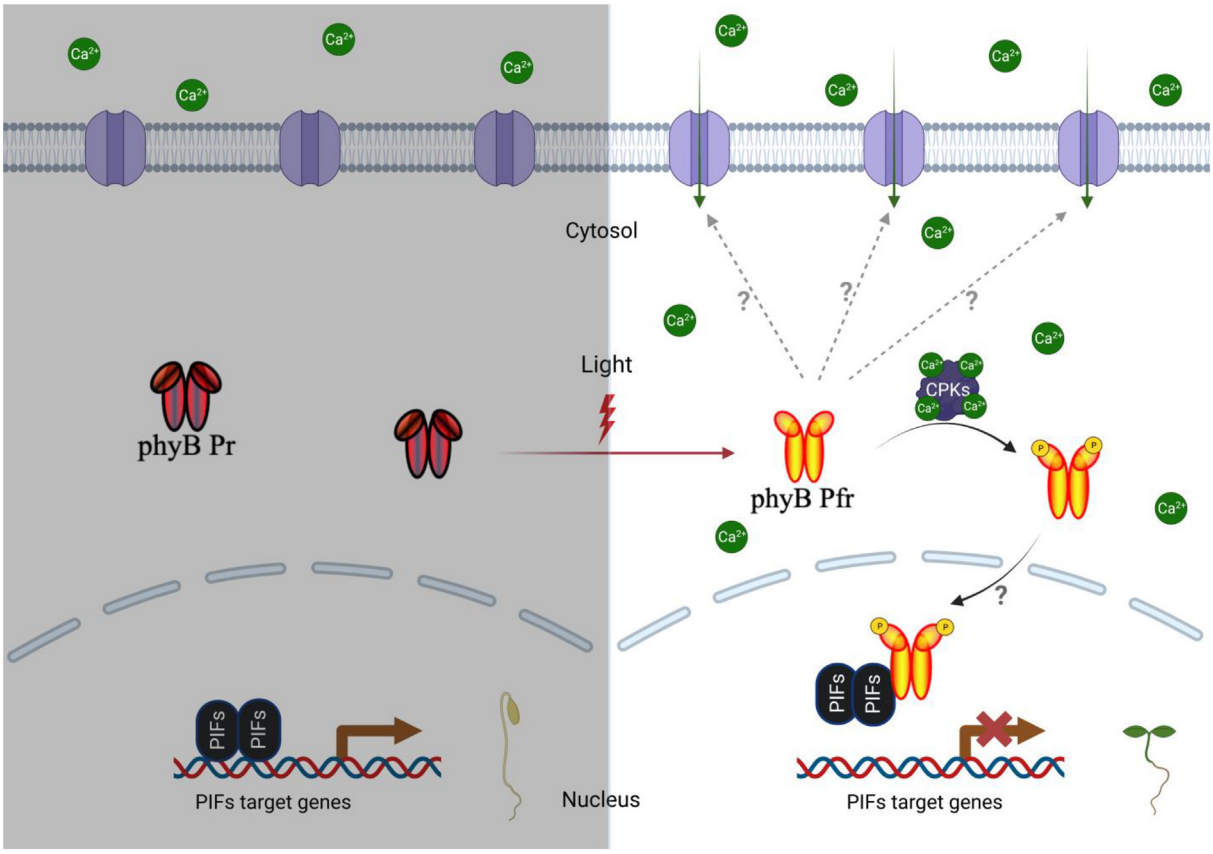

Fig. 1. In dark condition (left side), phyB is in inactive (Pr) form and stays in the cytosol and nuclear-localized phytochrome interacting factors (PIFs) bind to the promoter regions of PIF-target genes to regulate their expression, inhibiting photomorphogenesis. After light exposure (right side), phyB converts into active Pfr form and induces Ca^{2+} influx, which activates two CPKs (CPK6/12) to phosphorylate phyB. Phosphorylated phyB translocates into the nucleus to promote PIFs degradation and photomorphogenesis. This model is created with BioRender.com.

unmasks the NLS in the Pfr form of phyB [9,10]. However, the exact sequence acting as NLS has not been identified yet. In addition, phyB interaction with the suppressor of phyA-105 (SPA) and PIF family of proteins might mediate phyB's nuclear import. However, these interaction-mediated nuclear imports of phyB might play a minor role, if any. Zhao et al. [5] showed that the increased $[Ca^{2+}]$-cyt activated two calcium-dependent protein kinases, CPK6 and CPK12, which interact with phyB and phosphorylate phyB at S80 and S106 in a light- and Ca^{2+}-dependent manner to promote phyB nuclear translocation. By observing different variants of YFP-tagged phyB, including phyB-WT, phyB-2A (S80A and S106A), and phyB-2D (S80D and S106D) localization, they found phyB-WT and phyB-2D were mainly distributed in the nucleus in response to red light. However, phyB-2A was retained in the cytoplasm. These data suggest that S80/S106 phosphorylation is essential for light-induced phyB nuclear translocation. In addition, phyB-WT and phyB-2D can fully rescue the photomorphogenic defects of *phyB-9* mutant. However, the morphology of the phyB-2A transgenic plants is still similar to that of *phyB-9* mutant. In summary, phyB nuclear translocation is important for phyB function as has been shown over two decades ago [11,12] and S80/S106 phosphorylation mediates phyB nuclear import. Previous studies showed multiple phosphorylation sites are present at the N-terminus (from amino acids 80 to 120) of phyB, which was phosphorylated preferentially after light exposure [13]. Different phosphorylation sites show different effects on phyB activity and function. One well-studied light-induced phosphorylation site is tyrosine 104 (Y104) at phyB N-terminus [13]. The phosphorylation of Y104 exhibits a negative effect on phyB's activity because the phosphomimic version, phyBY104E failed to rescue phyB mutant phenotype and bind to PIF3. The other one is S86, which is phosphorylated even in dark condition. phyB-S86A and phyB-S86D don't affect the stability and photoconversion of phyB. However, light-independent relaxation of phyB-S86D Pfr into Pr is strongly enhanced, which attenuates the red-light-induced nuclear import of phyB [14]. Thus, phosphorylation at different sites at the N-terminus of phyB has distinct impacts on phyB function.

Photobody formation. After red light exposure, phyB moves into the nucleus and forms subnuclear speckles or bodies, named photobodies (PBs) [15]. phyB-mediated PBs' formation is a dynamic process. With increasing red-light intensity, phyB-GFP will form different PB patterns: diffuse nuclear localization; small photobodies only, both small and large photobodies; and finally large photobodies only. Interestingly, the phyB-GFP localization and PBs pattern are positively correlated with the degree of hypocotyl inhibition in response to red light, which suggests that the PBs patterns are important for phyB function [15]. The PB formation and patterns of phyB are affected by many factors of which one is phyB's phosphorylation status. Zhao et al. [5] found that phyB-GFP in *cpk6/cpk12* background fails to translocate into the nucleus and form the PBs after red light exposure during the dark-to-light transition. Thus, CPK6/12 are two important factors to regulate phyB phosphorylation and nuclear translocation. However, under prolonged red-light exposure, phyB-GFP in the *cpk6/cpk12* background

can translocate into the nucleus and form the PBs, but it only forms the small PBs as compared with the large PB in the wild-type background. In the YFP-tagged phyB localization assays, they found the phyB-2A mainly retained in the cytoplasm, even after 2 h of red-light exposure. However, phyB-WT and phyB-2D can translocate into the nucleus and form PBs. Interestingly, when NLS was added to phyB-2A, the authors observed strong phyB-2A-NLS nuclear localization and PBs formation, like phyB-WT-NLS and phyB-2D-NLS. In addition, all the variants of phyB with NLS can fully rescue *phyB-9* mutant phenotypes. These data suggest that S80/S106 phosphorylation regulates phyB nuclear translocation without affecting phyB's activity. The other light-induced phosphorylation site that showed effects on phyB PBs pattern is tyrosine 104 (Y104) of phyB [13]. Phosphomimic mutant, phyB (Y104E) failed to form stable PBs even though it still exhibits normal photochemistry *in vitro*. Researchers also focused on identifying the components of the PBs to understand PBs formation and function. A recent study shows that in phyB PBs there are two classes of proteins [16]. The first class is proteins that directly interact with phyB and localize into PBs, including constitutively photomorphogenic 1 (COP1)/SPA1, phyC/phyE, photo periodic control of hypocotyl 1 (PCH1)/PCH1-like (PCHL), PIF3/PIF4/PIF7, early flowering 3 (ELF3), and tandem zinc-finger-plus3 (TZP); and the second class includes proteins that interact and colocalize with the first class of proteins to PBs. In the second class of proteins, heat shock protein 70 (HSP70-1/HSP70-2) and topless (TPL)/TPL-related proteins (TPR1/TPR3/TPR4) are PCH1-requiring components to localize to phyB PBs and photoregulatory protein kinases (PPK1 to PPK3) are PIF3-requiring components to localize to phyB PBs. With the changes in light conditions, phyB might change its phosphorylation status to interact or incorporate different components in the PBs to regulate photomorphogenesis.

Future challenges. While Zhao et al. [5] enhanced our understanding of the light-induced nuclear translocation of light-stable phytochromes, especially phyB, several interesting questions remain unanswered. First, how phyB mediates Ca^{2+} influx? Does phyB directly interact with and activate Ca^{2+} channels causing Ca^{2+} influx or phyB needs other components? In dark-grown plant cells, phyB is mainly localized in the cytosol close to the membranes due to the presence of large vacuoles in plant cells. It is possible that phyB directly activates a Ca^{2+} channel. Second, which Ca^{2+} channel(s) mediate(s) the red-light induced [Ca^{2+}] increase? Is it the same channel mediating the blue light-induced [Ca^{2+}] increase? If yes, how do plants distinguish the [Ca^{2+}] increases to exhibit specific responses to red light and blue light? Third, the kinetics of the cytosolic Ca^{2+} increase and phyB nuclear translocations do not match. The cytosolic Ca^{2+} increase peaks within 30 s and levels off within 2 min. However, the phyB nuclear translocation is delayed by hours (2–4 h) under red light. Is the delay necessary to phosphorylate sufficient amounts of phyB for nuclear import or other factors involved in this process? Fourth, does Ca^{2+} has any other signaling roles in addition to regulating phyB nuclear translocation as originally proposed? Fifth, how is phosphorylated phyB imported into the nucleus? For phyA, the interaction between photoactivated phyA and FHY1/FHL is necessary for nuclear import [9]. Is it possible that other factors preferentially interact with the phosphorylated form of phyB and co-transport phyB into the nucleus as proposed previously? Sixth, what other kinases are involved in phosphorylating phyB at S80 and S106? phyB S80 and S106 phosphorylation controls phyB nuclear translocation in a general way. However, CPK6/12 mainly function during the dark-to-light transition. Thus, there may exist other kinases that phosphorylate phyB at S80 and S106 to control phyB nuclear translocation. The PPK kinases associate with phyB through PIF3, although PPKs have not been shown to directly phosphorylate phyB [17]. The identification of other kinases that phosphorylate phyB and answers to many of the above questions will provide new insights into a better understanding of how phyB regulates photomorphogenesis.

Conflict of interest

The authors declare that they have no conflict of interest.

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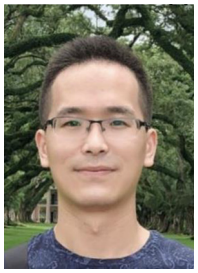

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