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SUMMARY STATEMENT

Phytochromes sense changes in the ratio and intensity of R and FR content of sunlight and by initiating/controlling a complex signaling network regulate nearly all aspect of plant growth and development. Recent research revealed exciting new aspects at molecular level how these photoreceptors function, uncovered the basic difference in the mode of action for the two major phytochrome species phyA and phyB and demonstrated that phyB is also function as thermosensor. This review summarizes and discusses the most important discoveries that opened new avenues for phytochrome-B related research

ABSTRACT (133 words)

The red/far-red light absorbing photoreceptors phytochromes regulate development and growth, and thus play an essential role in optimizing adaptation of the sessile plants to the ever changing environment. Our understanding of how absorption of a red/far-red photon by phytochromes initiates/modifies diverse physiological responses has been steadily improving. Research performed in the last five years has been especially productive, and led to significant conceptual changes about the mode of action of these photoreceptors. In this review we focus on the phytochrome B photoreceptor, the major phytochrome species active in light-grown plants. We discuss how its light-independent inactivation (termed dark/thermal reversion), post-translational modification, including ubiquitination, phosphorylation, sumoylation as well as heterodimerisation with other phytochrome species modify red-light-controlled physiological responses. Finally we discuss how photobiological properties of phyB enable this photoreceptor to function also as thermosensor.

INTRODUCTION

Light is a key environmental factor affecting almost every aspect of plants' life. It is not only the main source of energy for photosynthesis, but also acts as a developmental clue to harmonize growth with the ambient light environment, a

process termed photomorphogenesis. To alter the developmental program active in the dark (skotomorphogenesis) and thereby to ensure proper photomorphogenesis, plants have evolved a battery of photoreceptors. These sensors monitor the light spectrum, selectively absorb photons with different energies and translate light energy into biological signals to modulate the expression of thousands of genes that ultimately culminate in defined physiological responses. The widely used model plant *Arabidopsis thaliana* possesses the following photoreceptors: (i) the UV RESISTANCE LOCUS 8 (UVR8) absorbs ultraviolet B (Jenkins, 2014), (ii) the phototropins (Christie, 2007), the cryptochromes (Yu *et al.*, 2010) and ZEITLUPE type receptors (Kim *et al.*, 2007) are responsible for blue/UV-A perception, and (iii) phytochromes (phy) absorb red (R) and far-red (FR) light (Bae & Choi, 2008; Franklin & Quail, 2010).

Phytochromes exist in two interchangeable forms: the Pr form absorbs R light (λ_{max}=660 nm), whereas the Pfr form absorbs FR light (λ_{max}=730 nm). Phytochromes are synthesized in the Pr form in dark-grown seedlings, and absorption of a red photon induces conversion of Pr to Pfr, which is the biologically active phy conformer (Rockwell *et al.*, 2006). Pfr is rapidly converted back to Pr by FR light (photoreversion) or, in the absence of light, by dark reversion, also called thermal relaxation, (Mancinelli, 1994). This interconversion property of phytochromes allows these photoreceptors to function as R/FR-dependent molecular switches. The Arabidopsis phytochrome gene family contains five genes encoding phyA through phyE (Clack *et al.*, 1994). They are classified according to their stability: the type I is light-labile (phyA), whereas the type II phytochromes are light-stabile (phyB-E). phyA is the dominant phytochrome of dark-grown (etiolated) seedlings, but its amount decreases rapidly upon illumination. Type II phytochromes are the prevalent phytochromes of light-grown plants; among them phyB is the most abundant (Hirschfeld *et al.*, 1998; Sharrock & Clack, 2002). In photobiological terms three modes of action have been identified for phytochromes. Low fluence responses (LFRs) are typical R/FR reversible responses mediated nearly exclusively by type II phytochromes. Very low fluence responses (VLFRs) are triggered by extremely low quantities of light, mediated by phyA and not photoreversible, whereas the high irradiance responses (HIRs) produced by prolonged exposure to high-intensity light can be mediated by phyA or phyB (Nagy & Schafer, 2002).

PHYTOCHROME REGULATED PHYSIOLOGICAL RESPONSES

In Arabidopsis, phyA plays an important role in seedling establishment during the transition from skotomorphogenesis to photomorphogenesis. This and various other aspects of phyA signalling are discussed in the accompanying chapter in this issue. The switch to light-driven development, however, is not exclusively regulated by phyA. For example, regulation of germination and seedling de-etiolation (Su *et al.*, 2015) is mediated, beside phyA (Shinomura *et al.*, 1996), also by phyB and other type II phytochromes (Hennig *et al.*, 2002; Dechaine *et al.*, 2009; Lee *et al.*, 2012; Jiang *et al.*, 2016). The latter process results in the spectacular change of seedling morphology and manifests itself as inhibition of hypocotyl elongation, inducing opening of the cotyledon hook and expansion of the cotyledons (McNellis & Deng, 1995; Franklin & Quail, 2010; Kami *et al.*, 2010). In a light-dominated environment the action of type II phytochromes regulates production of functional photosynthetic apparatus, promotes chloroplast development (Chen *et al.*, 2010) alters photorespiration (Igamberdiev *et al.*, 2014),contributes to stomata development (Casson & Hetherington, 2014) and regulates stomata opening (Wang *et al.*, 2010). Apart from these responses phytochromes regulate (i) gravitropic orientation of roots and hypocotyls (Kim *et al.*, 2011; Hopkins & Kiss, 2012) and (ii) development of rosette, branching and apical dominance (Finlayson *et al.*, 2010; Franklin & Quail, 2010), thus, in principle, define the architecture of adult plants (Figure 1A).

Pr and Pfr forms of phytochromes have overlapping absorption spectra, thus these photoreceptors are also able to monitor the R/FR ratio of sunlight. This is of particular importance in natural habitats, when light is reflected or filtered through the leaves of neighbouring plants. Under a dense canopy the R/FR ratio of sunlight can dramatically change, because chlorophylls and carotenoids efficiently absorb R but not FR light, which results in a low R/FR ratio. Changes in R/FR ratio drastically modulate phytochrome signalling and trigger the so-called shade avoidance syndrome (SAS). This response, characterized by specific morphological changes such as leaf hyponasty, increased apical dominance, elongated petioles and early flowering, is of great importance for plants as it is essential for overgrowing competitors to optimize the efficiency of photosynthesis (Casal, 2012; Casal, 2013; Fraser *et al.*, 2016). SAS is mediated dominantly by phyB, but all members of the phy family are involved in the response, except for phyC (Franklin *et al.*, 2003). As stated above phyB as phyB

Pfr primarily mediates plant growth and development in response to changes in R/FR ratios and fluences in the ambient light environment. However, several lines of evidence indicate that phyB is also functioning under FR-HIR conditions when the majority of phyB molecules exist in their inactive Pr conformation. For example, it has been shown that seedlings overexpressing PHYB-GFP show pronounced etiolation phenotypes compared with the wild type counterparts under FR light (Wagner *et al.*, 1996; Casal *et al.*, 2000; Hennig *et al.*, 2001). This response can also be observed without the presence of phyA thus phyB inhibition of phyA function, under these circumstances, is not mediated by the direct interaction of these photoreceptors. More recently, it was also demonstrated that phyB is required for the proper nuclear accumulation of COP1 and SPA1 in FR, indicating that phyB can modulate the intracellular distribution of signaling components required for proper FR signaling (Zheng *et al.*, 2013). However, other factors such availability of nutrients (Short, 1999) also affect this response thus unravelling the precise molecular machinery for phyB action in FR will require further investigations.

Phytochromes, especially phyB, have also been shown to play a role in modulating signalling induced by biotic stress (herbivory) (Ballare, 2009), abiotic salinity (Carvalho *et al.*, 2011) and drought stress (Gonzalez *et al.*, 2012) and thermosensing (Franklin *et al.*, 2014; Johansson *et al.*, 2014; Quint *et al.*, 2016). Two recent papers which will be discussed in detail in this review, revealed the molecular mechanism underlying the role of phyB in integrating light and temperature induced signalling and established phyB as a *bona fide* thermosensor (Jung *et al.*, 2016; Legris *et al.*, 2016). All above described developmental/growth/stress responses similar to timing of flowering (Valverde *et al.*, 2004; Endo *et al.*, 2013) are also regulated by the circadian clock. A direct link between the action of red light receptors and the circadian clock has been already established. On the one hand all phytochromes, dominantly phyB, mediates transmission of light signals to the core clock mechanism (Devlin & Kay, 2000; Mas *et al.*, 2003; Huang *et al.*, 2016) on the other hand, most of the light-regulated processes are modulated by the clock, illustrating the complex mutual interactions of light and clock signalling pathways (Greenham & McClung, 2015) (Figure 1A).

STRUCTURE OF PHYTOCHROMES

All phytochromes have similar primary structures. The N-terminal domain of the apoprotein consists of the N-terminal extension (NTE), the PAS (PER-ARNT-SIM), the GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) and the PHY (phytochrome) domains (Figure 1B). The GAF domain cradles a linear tetrapyrrole chromophore (phytochromobilin) attached via a thioether bond to a conserved cysteine residue, and provides light sensitivity to the molecule (Nagatani, 2010). The C-terminal domain has regulatory functions, required for the dimerisation of the molecule; it contains two PAS domains as well as a motif related to histidine kinases (HKRD) (Nagatani, 2010; Vierstra & Zhang, 2011). Expressing the N-terminal domain of type II phytochromes alone proved that this domain is essential for light perception and signal transduction (Matsushita *et al.*, 2003; Oka *et al.*, 2008; Adam *et al.*, 2013). A recent report revealed the crystal structure of the N-terminal domain of Arabidopsis phyB, and provided additional insights into the conformational change underlying phyB signalling (Burgie *et al.*, 2014). The role of the different domains in mediating the interaction of phyB with signalling partners will be discussed in detail later in this review.

MOLECULAR MECHANISMS OF PHYB SIGNALLING

Light-induced translocation of phyB Pfr from the cytosol into the nucleus is an early and indispensable step in phyB signalling (Fankhauser & Chen, 2008; Klose *et al.*, 2015b). In contrast to phyA, which relies on the transport helper proteins FHY1 (FAR-RED ELONGATED HYPOCOTYL 1) and FHL (FHY1-LIKE), the mechanism of the light-dependent nuclear import of phyB is not comprehensively understood. PhyB nuclear import occurs independently of FHY1 and FHL (Hiltbrunner *et al.*, 2006). The C-terminal half of phyB lacking the chromophore binding domain is localized in the nucleus independently of light (Sakamoto & Nagatani, 1996; Matsushita *et al.*, 2003). Further experiments demonstrated that intramolecular interactions between the N-terminal and C-terminal domains of phyB occur preferentially in the Pr form and are weakened in the Pfr form. Based on these observations a molecular mechanism has been proposed, in which the conformational transition from the Pr to the Pfr form unmasks the nuclear localization motif in the C-

terminal domain to promote light-induced import of the photoreceptor into the nucleus (Chen *et al.*, 2005).

A more recent study offered an alternative interpretation of the above-mentioned findings. In a cell-free *in vitro* nuclear import system using isolated nuclei of the green alga Acetabularia, Pfeiffer *et al.* reconstituted the nuclear import of phyB only in the presence of transport factors that interact with phyB and carry an NLS (Pfeiffer *et al.*, 2012). Interestingly, neither the full-length nor the N-terminal or C-terminal half of Arabidopsis phyB alone was able to accumulate in the Acetabularia nuclei, indicating that phyB itself does not contain a functional intrinsic NLS-motif. Addition of PIF3 (PHYTOCHROME INTERACTING FACTOR 3) to the system induced nuclear import of phyB as well as of both phyB fragments. PIF3 was previously shown to interact with both the N- and C-terminal halves of phyB, whereby binding to the N-terminal domain was Pfr-dependent (Ni *et al.*, 1998; Ni *et al.*, 1999). In the Acetabularia system PIF3-mediated nuclear import of the C-terminal phyB fragment occurred independently of light, whereas that of the N-terminal fragment was clearly red-light–induced, indicating that the higher affinity of PIF3 to the Pfr-form is the reason for its light-dependent accumulation in the nucleus. The minimal requirements for a protein facilitating the nuclear import of phyB were narrowed down to a combination of a phyB-binding domain and an NLS, implying that any protein that interacts with phyB in a Pfr-specific fashion and contains an NLS could potentially mediate light-induced nuclear phyB import. This was further supported by the observation that nuclear import of phyB *in vivo* was impaired but not completely abolished in a *pifq* mutant lacking 4 of the PIF proteins (*pifq* = *pif1pif3pif4pif5*), which indicates that proteins other than PIFs are involved in the nuclear translocation of phyB (Pfeiffer *et al.*, 2012).

In the nucleus phyB controls seedling development by inhibiting two classes of repressors of photomorphogenesis: the COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC1)/ SPA (SUPPRESSOR OF phyA-105) complex and the PHYTOCHROME INTERACTING FACTORS (PIFs). These repressors by acting synergistically promote skotomorphogenesis, but are inhibited by photoactivated phytochromes allowing photomorphogenic development in light. In darkness the E3 ubiquitin ligase COP1 forms complexes with members of the SPA (SPA1-SPA4 in Arabidopsis) and PIF families and targets positive regulators of photomorphogenic growth for degradation by the proteasome (Xu *et al.*, 2014). Phytochromes inactivate

231 the COP1/SPA/PIF complex leading to exclusion of COP1 from the nucleus, resulting
232 in stabilization of its target proteins (Osterlund & Deng, 1998; Subramanian *et al.*,
233 2004; Pacin *et al.*, 2014) and degradation/inactivation of PIFs (Al-Sady *et al.*, 2006).
234 However, until recently the molecular mechanism underlying COP1/SPA inactivation
235 was not understood. It was demonstrated that phyA Pfr and phyB Pfr interact directly
236 with SPA1, and by reorganizing the COP1/SPA complex they promote
237 photomorphogenic development (Lu *et al.*, 2015; Sheerin *et al.*, 2015). These authors
238 show that photoactivated phyB competes with COP1 for SPA binding, thereby
239 disturbing the direct interaction between COP1 and SPA. Since SPA1 has been shown
240 to enhance the E3 ubiquitin ligase activity of COP1 in the complex (Seo *et al.*, 2003),
241 it is not yet clear whether disruption of the COP1/SPA complex by phyB directly
242 interferes with COP1 function on its target proteins, or rather eliminates the positive
243 effect of SPA1 on COP1 activity. The finding that photoactivated phytochromes
244 disrupt the direct interaction of COP1 and SPA provides a mechanistic model to
245 explain the fast inactivation of the COP1/SPA complex independently of the slow
246 process of COP1 exclusion from the nucleus.
247 Accumulation of phyB Pfr in the nucleus further initiates inactivation and
248 degradation of PIFs that act as negative regulators of photomorphogenesis as well.
249 PIFs are basic helix-loop-helix (bHLH) type transcription factors that regulate gene
250 expression to promote skotomorphogenesis (Duek & Fankhauser, 2005; Leivar *et al.*,
251 2008; Shin *et al.*, 2009). Photoactivated phyB directly interacts with PIFs and induces
252 their phosphorylation, ubiquitination and subsequent degradation by the proteasome
253 (Al-Sady, *et al.*, 2006; Shen *et al.*, 2007; Shen *et al.*, 2008; Leivar & Quail, 2011; Ni
254 *et al.*, 2013). Recently, the *in vivo* phosphorylation sites of PIF3 have been determined
255 during dark-to-light transition. Introducing multiple missense point mutations at the
256 phosphorylation sites stabilized the protein in light, whereas phospho-mimic
257 mutations promoted PIF3 degradation in the absence of light. These findings
258 supported the conclusion that light-induced phosphorylation of PIF3 is indeed
259 required for its subsequent degradation and for the negative feedback modulation of
260 phyB levels by PIFs in prolonged light (Ni *et al.*, 2013)
261 Recently Park *et al.* presented evidence that PIF degradation might not be the
262 primary mechanism by which phytochromes inhibit these repressors of
263 photomorphogenesis. The authors showed that the Pfr form of phyB was able to
264 inhibit the DNA binding capacity of PIF3, thereby preventing association to its target

promoters *in vivo* (Park *et al.*, 2012). These data indicated that phyB inhibition of PIF function requires interaction of these proteins but mediated by two different mechanisms, i.e. sequestration of PIFs and/or stimulation of their degradation. In this aspect we note that a recent work showed that phyB signalling in one cell, can efficiently initiate PIF degradation in other cells that do not contain phyB. (Kim *et al.*, 2016). This observation suggests that phyB initiated cell to cell signalling is involved in controlling activity of PIFs but (i) the chemical identity of the mobile signal(s), (ii) the molecular machinery mediating this type of degradation of PIF3 as well (iii) the overall impact of cell to cell communication on phyB signalling will remain to be elucidated.

Based on *in vitro* assays Martinez-Garcia *et al.* have proposed the hypothesis that light-dependent interaction with PIF3 recruits phyB to promoter elements of genomic targets, introducing the idea that phyB could be directly involved in the regulation of gene expression (Martinez-Garcia *et al.*, 2000). On the one hand it has been shown that phyA was able to associate with genomic DNA at promoter elements of numerous genes, many of them were identified as phyA-regulated target gene (Chen *et al.*, 2014). On the other hand a very recent report also demonstrated that phyB, similar to phyA can also be recruited to genomic promoter elements possibly via interaction with DNA-binding transcription factors (Jung *et al.*, 2016). These data indicate that individual and selective modulation of gene expression by phyA and phyB could play an important role in light induced signalling.

THE FUNCTIONAL ROLE OF DARK REVERSION IN PHYB SIGNALLING

PhyB acts as a light quality and quantity sensor and gradually controls photomorphogenic development depending on the light conditions. Analyses of phyB overexpression lines demonstrated that the light sensitivity of phyB-mediated photomorphogenic responses depends on phyB abundance (Wagner *et al.*, 1991; Rausenberger *et al.*, 2010). More precisely, the number of physiologically active Pfr molecules quantitatively determines the signalling efficiency of phyB. Since the absorption spectra of Pr and Pfr overlap considerably, a dynamic photoequilibrium between the Pfr and the Pr forms is established depending on the wavelength. The Pfr form has a higher energy state than the Pr form and is thermally unstable. Thus relaxation of Pfr into Pr can occur in a light-independent fashion (therefore it is also

termed dark reversion), but displays a strong temperature dependency (Schäfer & Schmidt, 1974; Hennig & Schäfer, 2001; Klose *et al.*, 2015a). A fast dark reversion process is able to compete with the light reaction of Pr-to-Pfr formation under non-saturating light conditions, leading to steady state Pfr levels lower than the photoequilibrium (the maximal relative Pfr level established depending on the light quality). Consequently, photoconversion and dark reversion determine the steady state level of the active Pfr conformation, enabling dynamic light quality and quantity sensing.

The PAS-GAF-PHY domains of Arabidopsis phyB N-terminal (photosensory module, PSM) recombinantly expressed in *E. coli* and reconstituted with phytochromobilin as chromophore exhibited efficient Pfr-to-Pr thermal reversion *in vitro* with a half-life of about 110 min, indicating that dark reversion is a property of the phytochrome molecule (Zhang *et al.*, 2013; Burgie *et al.*, 2014). In contrast, dark reversion of full-length phyB expressed in yeast and reconstituted with phycocyanobilin as chromophore showed very rapid initial dark reversion, but did not revert completely back to Pr (Eichenberg *et al.*, 2000; Sweere *et al.*, 2001). More recent *in vivo* studies, however, revealed that phyB Pfr reverts almost completely to Pr within 4 h of darkness, corresponding to an overall half-life of 60 min (Sweere *et al.*, 2001; Rausenberger *et al.*, 2010; Klose *et al.*, 2015a). Taken together, these studies indicate that in addition to being an intrinsic property of the phytochrome molecule, dark reversion is modulated by various external factors as well as intra- and intermolecular interactions.

Mutations altering conserved residues surrounding the chromophore in the phyB protein were shown to affect Pfr-to-Pr dark reversion differentially without impairing photoconversion. The Arg352Ala substitution stabilized Pfr against thermal reversion, whereas Arg322Ala caused a substantially faster dark reversion of purified recombinant PSM of phyB *in vitro* (Zhang *et al.*, 2013). Arabidopsis *phyB* mutant seedlings expressing the full-length phyB[Arg352Ala] showed normal phyB signalling under high fluence rates of red light and in white light, but were hypersensitive under low fluence rates, suggesting that thermal reversion impacts phyB action when light conditions are limiting. Consistent with this conclusion, Oka *et al.* showed that the Arg322Gln substitution reduced responsiveness of Arabidopsis seedlings expressing the full-length mutant variant under intermittent red light pulses (Oka *et al.*, 2008).

The NTE domain of phyB has been shown to stabilize Pfr, and mutants lacking this domain exhibit accelerated dark reversion *in vitro* (Zhang *et al.*, 2013). The PHY domain contains a unique tongue-like structure that interacts with the GAF domain bearing the chromophore. This protrusion has been implicated in the transmission of conformational changes from the chromophore retained in the GAF domain to the PHY domain and consequently the whole molecule. Thereby the tongue was found to refold during transmission from Pr to Pfr from a beta-strand to an alpha-helix (Takala *et al.*, 2014). Mutations in this tongue region of the PHY domain of phyB, e.g. Arg582Ala, Gly564Glu (*phyB-401*) have been described leading to a dramatically enhanced thermal stability of the Pfr form resulting in strong hypersensitivity of seedlings grown under weak red light (Kretsch *et al.*, 2000; Ádám *et al.*, 2011; Zhang *et al.*, 2013). In addition, the Glu812Lys mutation (*phyB-101*) in the second of the two PAS domains in the C-terminal of phyB (Figure 1B) caused enhanced dark reversion in combination with a loss-of-function phenotype, demonstrating that protein domains that are more distant from the chromophore could also affect Pfr thermal stability (Elich & Chory, 1997). It would be interesting to investigate whether other phyB loss-of-function mutants might be affected in dark reversion as well.

Phytochromes form dimers *in vivo,* and dimerization has been shown to be important for their physiological function (Matsushita *et al.*, 2003). Consequently, phytochrome dimers can exist in three different states: Pr-Pr, Pfr-Pr, and Pfr-Pfr. A recent study demonstrated that the different dimer species of phyB indeed exhibit differential kinetic properties that are fundamental for the mode of phyB action (Klose *et al.*, 2015a). Already in 1987 it was proposed that dark reversion has different kinetics for Pfr-Pfr and Pfr-Pr dimers based on *in vivo* observations (Brockmann *et al.*, 1987). This was supported by the finding that recombinant Pfr-Pr dimers expressed in yeast showed fast and complete dark reversion in contrast to Pfr-Pfr dimers that remained more stable (Hennig & Schäfer, 2001). Klose *et al.* (2015a) combined *in vivo* measurements and mathematical modelling to demonstrate that Pfr-Pr heterodimers and Pfr-Pfr homodimers exhibit extremely different dark reversion kinetics, with Pfr-Pr dark reversion being almost 100-fold faster as compared to Pfr-Pfr. These findings lead to the conclusion that in Arabidopsis the phyB Pfr-Pr heterodimer pool undergoes fast dark reversion, resulting in reduced amounts of active phyB, particularly under light conditions that favour the generation of Pfr-Pr

heterodimers, e.g. lower light intensities or wavelengths above 690 nm. As the physiological phyB function is inhibited under such light conditions, it was concluded that only the Pfr-Pfr homodimers in the nucleus are able to initiate phyB-mediated light signalling (Klose *et al.*, 2015a). In other words, the slow dark reversion of the Pfr-Pfr homodimer determines the persistence of phyB signalling after transfer to darkness, whereas the extremely fast dark reversion of the Pfr-Pr heterodimer competes efficiently with the Pr to Pfr photoconversion, reducing the Pfr levels under non-saturating irradiation.

The precise nature of the fast Pfr-Pr dark reversion process needs to be determined. It is possible that the thermal stability of the Pfr-Pr dimer is affected when only one of the two subunits has undergone the conformational change from Pr to Pfr. Alternatively, the Pfr form of phyB could be stabilized by interactions with other proteins, for example ARABIDOPSIS RESPONSE REGULATOR 4 (ARR4), and such stabilization may work more efficiently for the Pfr-Pfr homodimer (Sweere *et al.*, 2001). Phosphorylation of specific amino acids, especially that of Ser86 residing in the N-terminal domain of phyB can also modify dark reversion and red light signalling by an ARR4-independent mechanism (Medzihradszky *et al.*, 2013); this is discussed in more detail in the following section.

Upon light irradiation, phyB associates within discrete subnuclear structures named photobodies (PBs) (Chen *et al.*, 2003; Fankhauser & Chen, 2008). Light conditions establishing high Pfr levels promote the formation of large PBs *in vivo* (Trupkin *et al.*, 2014; van Buskirk *et al.*, 2014). Thus it has been proposed that these PBs function in stabilizing phyB Pfr, which allows phyB to continue controlling the level of PIFs and suppressing hypocotyl growth after light-dark transfer (Rausenberger *et al.*, 2010; van Buskirk *et al.*, 2014; Klose *et al.*, 2015a). Very recently it was shown that PCH1 (PHOTOPERIODIC CONTROL OF HYPOCOTYL 1), a protein that is associated with the Evening Complex in Arabidopsis, binds phyB in a red-light-dependent manner and co-localizes with phyB into PBs (Huang *et al.*, 2016). With the need to be verified experimentally, the authors presented a model, in which binding of PCH1 to phyB after light exposure slows dark reversion of phyB Pfr, thereby extending the lifetime of phyB-containing large PBs (Huang *et al.*, 2016). A correlation between dark reversion rates, PB formation and stability has been observed previously: mutant phyB molecules exhibiting accelerated dark reversion often failed to localize to PBs under normal light conditions or required higher fluence rates of red light, whereas

mutants with slower dark reversion accumulated into PBs even under weak fluence rates (Ádám *et al.*, 2011; Medzihradszky *et al.*, 2013; Zhang *et al.*, 2013).

POST-TRANSLATIONAL MODIFICATIONS OF PHYB

Ubiquitination

The E3 ubiquitin ligase COP1 was shown to interact with the N-terminal fragment of phyB, it was capable to ubiquitinate the photoreceptor and ubiquination of phyB was stimulated by the presence of PIF3 in these *in vitro* assays (Jang *et al.*, 2010). More recently, mass-spectrometry analysis of proteins co-purified with PIF3 from Arabidopsis identified components of a Bric-a-Brack/Tramtrack/Broad (BTB)-Cullin3-type E3 ubiquitin ligase as red-light-specific PIF3-interacting proteins (Ni *et al.*, 2014). Interestingly, the two highly conserved BTB proteins LRB1 (Light-Response-BTB1) and LRB2 had been previously shown to be required for proteasomal phyB degradation (Christians *et al.*, 2012) Ni *et al.,* however, could show that PIF3 phosphorylation triggers recruitment of LRB E3 ubiquitin ligases to the PIF3-phyB complex, whereupon LRBs promote polyubiqutination and degradation of both PIF3 and phyB *in vivo* (Ni *et al.*, 2014). The proposed PIF3-phyB co-degradation model provides a mechanistic explanation for phyB-induced PIF3 degradation and concurrent signal attenuation by photoreceptor degradation (Zhu & Huq, 2014). PIF3 degradation is about 50-fold faster as compared to phyB degradation. The strongly different degradation kinetics of PIF3 and phyB were explained by the different protein levels in seedlings, where phyB is much more abundant than PIF3, which was supported by the fact that overexpression of PIF3 enhanced phyB degradation (Ni *et al.*, 2013; Ni *et al.*, 2014). Whereas phyB degradation in red light was completely abolished in an *lrb123* triple mutant, PIF3 degradation was only slowed down. The results are compatible with the hypersensitive phenotype of *lrb123* in light (Christians *et al.*, 2012) that is consistent with the observed higher phyB abundance in light, but not with a defective PIF3 degradation (Ni *et al.*, 2014). These observations suggest that the main function of LRBs is signal attenuation by photoreceptor degradation, and that there is partial functional redundancy between the LRBs and other unknown E3 ligases for PIF3 degradation.

Phosphorylation

Early studies performed using purified oat and maize phytochromes indicated that phytochromes have autophosphorylation activity whereas sequence comparison showed that the C-terminal domain of phytochromes contains a region homologous to bacterial histidine kinases (Schneider-Poetsch *et al.*, 1991). Research performed to clarify how and to what extent (reversible) phosphorylation modulates phyA action produced plenty of data (Kim *et al.*, 2004; Ryu *et al.*, 2005; Han *et al.*, 2010), yet until very recently the significance of the postulated kinase activity of phyA (Yeh & Lagarias, 1998; Fankhauser *et al.*, 1999) was debated (for details see accompanying review article in this issue). Here we only note that a very recent report identified the kinase domains of various plant phytochrome species including oat and Arabidopsis phyA, and demonstrated that this region is critical for ATP-binding (Shin *et al.*, 2016). These authors also provided convincing evidence that perturbation of this region inhibited phosphorylation of PIF3 by oat phyA *in vitro,* and confirmed in transgenic plants that the kinase activity of phyA is critical for efficient light-induced signalling.

In contrast to phyA, our knowledge about the phosphorylation of phyB is rather limited, although it was shown that (i) PAPP5 and PAPP2c (PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE) proteins bind to the Pfr form of phyB, (ii) their null mutants show reduced responses in R light, and that (iii) phyB is phosphorylated *in vitro* and also interacts with the protein phosphatase PAPPC2 (Ryu *et al.*, 2005; Phee *et al.*, 2008). These observations suggested that phosphorylation of the photoreceptor attenuates light signalling. More recent studies identified a number of phosphorylated residues of phyB (Medzihradszky *et al.*, 2013; Nito *et al.*, 2013). Medzihradszky *et al*. demonstrated that the Ser86 located in the N-terminal domain of the protein is phosphorylated *in planta*. The phospho-mimic phyB[Ser86Asp] mutant shows fast dark reversion, and thereby decreases the amount of phyB Pfr. The low Pfr level of the mutant phyB slows down the import of the receptor into the nucleus and limits its interaction with PIF3; in other words, phosphorylation of phyB effectively attenuates light signalling. Consistent with this conclusion the non-phosphorylatable phyB[Ser86Ala] mutant displays slower dark reversion *in vitro* and *in planta,* thus transgenic plants expressing this mutant exhibit hyperactive responses including inhibition of hypocotyl elongation, cotyledon expansion, shade avoidance and flowering, particularly under low light intensity conditions, where Pfr amount is limiting (Medzihradszky *et al.*, 2013; Hajdu *et al.*,

2015). Besides Ser86, work performed by Nito *et al*. revealed nine further phosphorylated amino acid positions in Arabidopsis phyB (Ser84, Tyr89, Tyr90, Tyr91, Ser94, Ser95, Tyr104, Ser106, Tyr113). These amino acids are located in a cluster named PCSM motif (Phosphorylation Cluster of Signaling Modulation) spanning from Ser84 to Tyr113 (Figure 1B) and are conserved evolutionarily, indicating their general regulatory importance (Nito *et al.*, 2013). The phosphorylation of each identified amino acid negatively regulates phyB signalling, but among them Tyr104 has the most pronounced phenotype. Tyr104 is phosphorylated after light exposure, and the phospho-mimic mutant phyB[Tyr104Glu] possesses no light signalling activity at all, whereas the non-phosphorylated phyB[Tyr104Phe] shows enhanced activity as compared to wild-type phyB (Nito *et al.*, 2013). Similarly to Ser86, phosphorylation of Tyr104 also attenuates phyB signalling, presumably also by accelerating dark reversion. These data suggest that this domain of the molecule could be a "hot-spot", where Pfr stability is regulated according to the actual light conditions.

Beside the PCSM domain, phyB was reported to be autophoshorylated at unknown sites within its NTE domain (1-100) by (Phee *et al.*, 2008) *in vitro* and at the Ser596, Tyr601, Ser977, Ser1163 residues *in planta* (Nito *et al.*, 2013). These latter amino acids were phosphorylated in the dark and in the light as well, and the function of these modifications is not known (Nito *et al.*, 2013). A very recent study demonstrated that phyB and phyD – similarly to phyA – have kinase activity, autophosphorylate and can phosphorylate PIF3 *in vitro.* The amino acids critical for ATP-binding reside in the N-terminal domain of phyA (1-651) (Shin *et al.*, 2016). The equivalent N-terminal domain of phyB appears to play a significant role in regulating dark reversion (see dark reversion chapter above). Thus we speculate, although the ATP-binding site and kinase activity of phyB is yet to be identified *in planta,* that modulation of dark reversion by reversible autophosphorylation and/or phosphorylation of phyB by other kinases as well its ability to phosphorylate other proteins must be harmonized.

SUMOylation

Reversible, covalent conjugation of Small Ubiquitin-Like Modifier (SUMO) molecules to target proteins regulates protein activity and different cellular responses in eukaryotic cells. The conjugation and removal of SUMO is performed by a small set of enzymes, which have conserved structure throughout different organisms

(Miura & Hasegawa, 2010; Hickey *et al.*, 2012; Novatchkova *et al.*, 2012). The sumoylation state of the protein pool depends on various factors (including stress, developmental state, hormonal signalling etc.), furthermore numerous plant SUMO substrates were identified in the past few years (Elrouby & Coupland, 2010; Miller *et al.*, 2010).

Recently it was reported that phyB is sumoylated *in planta*, the SUMOylated form of phyB accumulates to high levels when the receptor is in the Pfr form, and phyB SUMOylation is reversible (Sadanandom *et al.*, 2015). It was also demonstrated that the target lysine of SUMO conjugation is located in the C-terminal domain of phyB. The sumoylation of the mutant phyB[Lys996Arg] is negligible, and the transgenic plants expressing this receptor are hypersensitive in R light. This phenotype could be - at least partly - explained by the reduced binding of the SUMOylated phyB to the negative regulator transcription factor PIF5. Thus these authors concluded that SUMOylation of phyB attenuates light signalling by reducing the formation/stability of the phyB-PIF complexes (Sadanandom *et al.*, 2015). Consistent with its reversibility, the SUMOylation level of the phyB pool appears to be regulated at least partly by the concerted action of OVERLY TOLERANT TO SALT (OTS) 1 and 2 SUMO proteases. OTS1 binds directly to phyB and removes the SUMO from the protein. Compared to wild-type plants, the accumulation level of the SUMOylated phyB pool is higher in the *ots1ots2* mutant plants, which show a hyposensitive photomorphogenic phenotype in R light (Sadanandom *et al.*, 2015). It remains to be seen if SUMOylation – similarly to phosphorylation – also targets, beside phyB, other phytochrome species and/or down-stream signalling components.

HETERODIMERIZATION OF TYPE II PHYTOCHROMES

For many years, after discovering that phyA purified from dark-grown oat seedlings exists primarily as dimer (Lagarias & Mercurio, 1985) it was generally agreed that the type II phytochromes are also active as homodimers. However, two seminal papers (Sharrock & Clack, 2004; Clack *et al.*, 2009) changed this view. First, these authors demonstrated that Arabidopsis contains multiple species of both homodimeric and heterodimeric phyB and phyD phytochromes, but phyA is present only as a homodimer and does not form heterodimers with any other phytochrome species. Next, they reported that phyC and phyE do not homodimerize, but heterodimerize

with phyB and phyD and that the expression/activity of phyC in a *phyBphyD* mutant, where none of its dimerization partners was present, dropped dramatically (Clack *et al.*, 2009). Clack *et al*. also showed that not only phyB but phyC and phyD, presumably as members of phyB/phyC and phyB/phyD heterodimers co-immunoprecipitate from seedling extracts with the PIF3 transcription factor in a R/FR-reversible manner (Clack *et al.*, 2009). Although direct interaction of phyC, phyD and phyE with PIF3 has not yet been detected i*n planta*, these results show that all phytochromes in homo- or heterodimeric forms appear to function through PIF-mediated pathways.

Two more recent reports demonstrated that (i) homodimers of the N-terminal fragments of all type II phytochromes were biologically active in the modulation of R-light-regulated photomorphogenesis (Adam *et al.*, 2013) and that (ii) heterodimers of the N-terminal domains of phyB/phyC, phyB/phyD, phyB/phyC, phyB/phyE etc. generated by using a synthetic biological approach showed slightly different phenotypic responses when compared phyB/phyB. For example, the phyB/ phyB[Cys357Thr] heterodimer containing the chromophore-less version of phyB was active in petioles and cotyledons, but not in hypocotyls (Liu & Sharrock, 2013). Taken together, the above findings suggested that the formation of such type II heteromeric photoreceptors increases the potential complexity of R/FR light sensing, for example phyC might signal only as heterodimer, yet the question of how and to what extent remained to be answered. Just recently by using a bottom-up assembly of phytochrome network Sanches-Lamas *et a.,* provided more insight into the biological function of phytochrome heterodimerisation (Sanchez-Lamas *et al.*, 2016). In this elegant study the authors first expressed each of the five phytochromes in the quintuple *phyAphyBphyCphyDphyE* mutant and then created lines expressing pairwise these phy genes in all possible combination. Analysis of this set of mutant plants revealed many new features of the phytochrome network and demonstrated among others that phyB alone is sufficient to confer full hypocotyl, germination responses to R and repress flowering but phyB and phyC co-action is needed to confer responsiveness to photoperiod. These findings indicate that phyB/phyB homodimers are mediating responses to light quality whereas phyB/phyC heterodimers are essential for the manifestation of a proper photoperiodic response. These authors also showed that association of phyB to nuclear bodies also modified by phyC and concluded that phyB/phyC heterodimers are probably active for longer periods in

darkness which could be an important factor to repress flowering and hypocotyl elongation especially under short-day conditions. In addition, on the one hand they also clarified individual contribution of phyD and phyE to a variety of light controlled responses, for example they showed that phyE strongly repressed flowering but had little effect on controlling hypocotyl growth. On the other hand they also uncovered synergestic and antagonistic effects of phytochromes in controlling germination and flowering and hypothesized that at least part of these responses is mediated by heterodimers of the various phytochrome species. More importantly they have suggested by analysing a large number transgenic lines expressing these phytochromes at different level that the role of the individual phytochrome species is determined by the intrinsic properties of these photoreceptors (such as ability to heterodimerize, photochemical features, interaction with signaling partners etc.) rather than by the expression level or patterns. Nothwithstanding these very convincing data, however, it is also true that even a slight reduction of the phyB expression level significantly alters red light responsiveness, indicating that modification of the ratio of phyB/phyB homodimers by other type II phytochromes could be an important factor. At present, the molecular mechanism regulating/limiting homodimerization and/or heterodimerization of phyB with other type II phytochromes is not known, nor is it known how these phyB-containing heterodimers function, i.e. whether they regulate the expression of genes at least partly different from those regulated by homodimers. Given the importance of dark reversion and post-translational modifications of phyB in regulating red light-induced signalling, we speculate that these could also be affected by heterodimerization with phyC, phyD and phyE.

ROLE OF PHYB IN TEMPERATURE SENSING/ INTEGRATION OF LIGHT AND TEMPERATURE SIGNALING

A growing amount of findings has led to the recognition that light and temperature signals are integrated by multiple mechanisms (Franklin *et al.*, 2014; Johansson *et al.*, 2014; Quint *et al.*, 2016). The morphological changes induced by high ambient temperature, collectively summarized as thermomorphogenesis, include the promotion of elongation growth which parallels the response to unfavourable light conditions in vegetational shade (Casal, 2012). Interestingly, PIF4, a positive regulator of the shade avoidance response, was identified as central component of ambient temperature

signalling (Koini *et al.*, 2009). PIF4 functions in regulating phytohormone biosynthesis and signalling. Expression of *PIF4* is controlled by the circadian clock through repression by the Evening Complex but is increased by high temperature (Nozue *et al.*, 2007; Nusinow *et al.*, 2011). On the posttranslational level PIF4 activity and abundance is controlled by phyB. PIF4 interacts specifically with light activated phyB leading to its phosphorylation and subsequent degradation (Lorrain *et al.*, 2008). Two very recent complementary studies have demonstrated that phyB directly participates in temperature perception based on the temperature dependency of its kinetic properties (Jung *et al.*, 2016; Legris *et al.*, 2016). Although it has been described previously that dark reversion is strongly temperature dependent (Schäfer & Schmidt, 1974; Hennig & Schafer, 2001; Klose *et al.*, 2015a) the two papers highlighted the role of dark reversion in plant temperature responses considering also the differential properties of the phyB dimers.

Jung *et al.* (2016) showed that high temperature accelerates the phyB Pfr decay during night time which is based on the temperature sensitivity of the slow dark reversion process of the Pfr-Pfr homodimer. Active phyB was shown to associate in a temperature dependent manner with promoters of genes that are also targeted by PIFs. Faster phyB dark reversion at higher temperature correlated with the loss of phyB occupancy at target gene promoters leading to the conclusion that phyB could transmit temperature information by inhibiting PIF activity through direct binding at target promoters. These findings were supported by extensive gene expression analyses showing that the warm temperature transcriptome is specifically affected by phytochrome activity during nighttime. Phytochrome null mutants displayed a constitutive warm temperature transcriptome even at low temperatures whereas in the constitutively active phyB[Tyr276His] allele the warm temperature transcriptome was constitutively repressed during night.

Legris *et al.* (2016) showed that temperature regulation of phyB Pfr levels is effective not only at night but also during the day. In light, the steady state levels of phyB Pfr are determined by the photoconversion rates, depending on the light quality and intensity, as well as by the fast dark reversion rate of the Pfr-Pr heterodimer (Klose *et al.*, 2015a). Using both, *in vitro* and *in vivo* spectroscopic assays, the authors demonstrated that the fast Pfr-Pr dark reversion rate of phyB is strongly sensitive to temperature (Legris *et al.*, 2016). This is particularly obvious under low light conditions, where Pr to Pfr photoconversion is slower. Under such conditions the Pfr-

Pr heterodimers are more abundant compared to higher light intensities and might undergo dark reversion rather than absorbing another photon to become Pfr-Pfr. High temperature favors the dark reversion reaction thereby reducing the Pfr steady state levels especially at low light conditions. PhyB containing nuclear bodies reflect the status of phyB since they are mainly composed of Pfr-Pfr homodimers. As a proxy for temperature effects on Pfr-Pfr levels Legris et al. (2016) quantified the nuclear body sizes of wild-type phyB and two phyB mutant alleles with suppressed thermal reversion (phyB[Tyr361Phe] and phyB[Arg582Ala]) (Zhang *et al.*, 2013) that are not sensitive to temperature changes for a range of different temperatures and light condition. Although they could not detect a straight correlation between temperature and nuclear body size for wild-type phyB, they observed a strong reduction in nuclear body size at temperatures higher than 20°C. By using a mathematical model describing the relation between Pfr-Pfr levels and nuclear body size they could show independently of the spectroscopic measurements that high temperatures decrease the apparent phyB Pfr-Pfr amount. Mathematical modeling of growth responses mediated by phyB, temperature and phyB-independent pathways further revealed that phyB-mediated temperature effects contribute significantly to growth regulation thereby showing largest effects at low irradiances (Legris *et al.*, 2016).

Taken together, these studies support the idea that phyB is physiologically responsive to perceive light and temperature signals at the same time indicating that phyB, in its active Pfr conformation, should also be defined as a temperature sensor.

CONCLUDING REMARKS

Phytochrome signalling is an extensively studied field of photobiology. After learning the basics of the receptors' photochemistry, we have greatly extended our knowledge about the molecular mechanisms of phytochrome action, with a special respect to the identification of phytochrome-interacting protein partners. More recent findings revealed the molecular machinery that mediates integration of phytochrome signalling not only with hormone-induced actions (de Lucas & Prat, 2014; de Wit *et al.*, 2016), but also those induced by various biotic and abiotic stresses (Ballare, 2014; Cortes *et al.*, 2016) and by temperature (Jung *et al.*, 2016; Legris *et al.*, 2016). It is predictable

that this trend will continue; however this review demonstrates that we still have a lot to learn about the phytochrome photoreceptors themselves.

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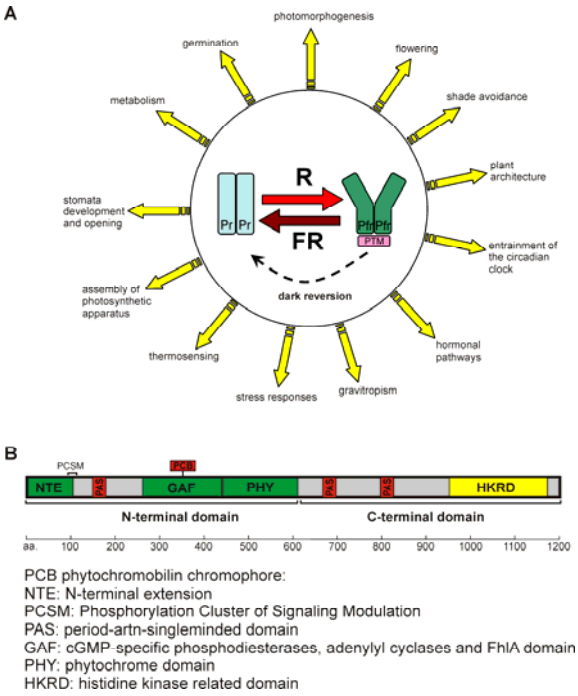
FIGURE



PCB phytochromobilin chromophore:
NTE: N-terminal extension
PCSM: Phosphorylation Cluster of Signaling Modulation
PAS: period-artn-singleminded domain
GAF: cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA domain
PHY: phytochrome domain
HKRD: histidine kinase related domain

Figure 1

A. Phytochrome B-controlled responses in Arabidopsis thaliana.
The ratio of available Pr and Pfr forms of phyB molecules are tuned by the intensity of red (R) and far-red (FR) light (photoconversion) together with the dark reversion. The Pr/Pfr dimers are not depicted to maintain clarity (see text for details). PTM indicates post-translational modifications of the Pfr form.
B. Schematic structure of the phyB monomer.