

Quantitative Aufklärung der Wechselwirkungen zwischen Phytochromen und Partnerproteinen

DISSERTATION

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David Golonka

aus *Haßfurt*

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Amtierender Dekan: Prof. Dr. Matthias Breuning

Prüfungsausschuss:

Prof. Dr. Andreas Möglich	(Gutachter/in)
Prof. Dr. Birte Höcker	(Gutachter/in)
Prof. Dr. Roland Marschall	(Vorsitz)
Prof. Dr. Frank Hahn	

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Abkürzungsverzeichnis

APA	<i>active phytochrome A binding</i>
APB	<i>active phytochrome B binding</i>
<i>At</i>	<i>Arabidopsis thaliana</i>
ATP	Adenosintriphosphat
bHLH	<i>basic helix-loop-helix</i>
BLUF	<i>sensor of blue light using flavin adenine dinucleotide</i>
BphP	Bakteriophytochrom
BV	Biliverdin
CA	katalytische Domäne
CBCR	Cyanobakteriochrom
DHp	<i>dimerization and phospho-accepting histidine</i>
<i>Dr</i>	<i>Deinococcus radiodurans</i>
<i>Ds</i>	<i>Discosoma species</i>
EYFP	<i>enhanced yellow fluorescent protein</i>
GAF	<i>cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA</i>
GFP	<i>green fluorescent protein</i>
HK	Histidinkinase
HKRD	<i>histidine kinase related domain</i>
HO	Hämoxygenase
Ilac	<i>light-activated adenylyl cyclase</i>
IR	Infrarot
LAPD	<i>light-activated phosphodiesterase</i>
LOV	<i>Light-oxygen-voltage-sensing</i>
NTE	<i>N-terminal extension</i>
(s)OPA	<i>(shortened) only phytochrome A binding</i>
PAC	<i>photoactivated adenylyl cyclase</i>
PAS	Per-ARNT-SIM
PCB	Phycocyanobilin
Pfr	fernrotlichtabsorbierender Zustand
PHY	<i>phytochrome-specific</i>
PhyA	Phytochrom A
PhyB	Phytochrom B
PIF	Phytochrominteraktionsfaktor
PPI	Protein-Protein Interaktion
Pr	rotlichtabsorbierender Zustand

PSM	photosensorisches Modul
PYP	<i>photoactive yellow protein</i>
PΦB	Phytochromobilin
REC	<i>response regulator receiver domain</i>
UV	Ultraviolett

Zusammenfassung

Die Wahrnehmung von Licht ist essenziell für viele Organismen, um sich an ihre Umgebung anzupassen. Zu diesem Zweck besitzen Lebewesen sensorische Photorezeptorproteine mit unterschiedlichster Lichtsensitivität. Photorezeptoren bestehen aus einem Sensormodul, das den Chromophor für die Lichtwahrnehmung enthält, und einem Effektormodul für die Vermittlung der biologischen Funktion. Aufgrund ihrer Sensitivität, der Reversibilität und der zeitlichen und räumlichen Präzision der kontrollierten Antworten sind sie daher ein gefragtes Werkzeug, um zelluläre Prozesse mit Hilfe von Licht zu steuern, eine als Optogenetik bezeichnete Technik. Phytochrome sind rot- und fernrotlichtabsorbierende Photorezeptoren, deren Signalzustände bimodular gesteuert werden können. Die von ihnen detektierten Wellenlängen besitzen eine hohe Eindringtiefe in biologisches Gewebe und sind daher von hohem Interesse für die Optogenetik.

In dieser Doktorarbeit wurden verschiedene Aspekte von Phytochromsystemen untersucht, wobei ein Hauptaugenmerk auf lichtgesteuerte Protein-Protein Interaktion, die von pflanzlichen Phytochromen eingegangen werden, lag. Die Wechselwirkung zwischen dem *Arabidopsis thaliana* Phytochrom B (PhyB) und seinen Interaktionsfaktoren (PIFs) ist die bekannteste rot- bzw. fernrotlichtabhängige Protein-Protein Interaktion. Nach Rotlichtbestrahlung erfährt AtPhyB strukturelle Veränderungen auf molekularer Ebene, die die Wechselwirkung mit PIF Proteinen erlauben. Nach längerer Zeit im Dunkeln oder nach Fernrotlichtbestrahlung geht die Interaktion jedoch wieder verloren. Trotz ihrer vielfältigen Anwendung ist überraschenderweise relativ wenig über die Interaktion dieser Proteine bekannt. So konnte in der Vergangenheit zwar bereits gezeigt werden, dass lediglich das photosensorische Modul von AtPhyB und das APB (*active phytochrome B binding*) Motiv der PIFs für die Wechselwirkung benötigt werden, jedoch ist nicht viel über die Interaktionsstärke und Dynamik bekannt. Aus diesem Grund wurden verschiedene biophysikalische und fluoreszenzspektroskopische Verfahren etabliert und angewandt, um die Interaktion von natürlichen und modifizierten PIF Proteinen mit dem rotlicht- bzw. fernrotlichtadaptierten Zuständen von AtPhyB zu untersuchen. Die gewonnenen Erkenntnisse eröffnen neue Einblicke in das APB Motiv von PIFs, liefern Dissoziationskonstanten für die Interaktion von PIF Proteinen mit den beiden Zuständen von AtPhyB und zeigen auf, dass die Interaktionsstärke durch die Assoziationsreaktion definiert wird.

A. thaliana besitzt noch weitere Phytochrome, jedoch ist zum aktuellen Zeitpunkt noch wenig über potenzielle Interaktionspartner bekannt. In einem kollaborativen Projekt wurde ein

Protein, benannt als OPA, mit unbekannter biologischer Funktion identifiziert, das lichtabhängige Protein-Protein Interaktion mit Phytochrom A (PhyA) eingeht und keine Wechselwirkung mit *AtPhyB* zeigt. In dieser Arbeit wurde die lichtabhängige Interaktion von *AtPhyA* mit OPA bzw. einer verkürzten OPA Variante untersucht.

Um die zuvor beschriebenen Wechselwirkungen ausüben zu können, verwenden pflanzliche Phytochrome den Chromophor Phytochromobilin, der nicht in anderen Organismen vorhanden ist. Für die Anwendung außerhalb von Pflanzen setzt dies die Versorgung mit geeigneten Chromophoren voraus, was die Einsatzmöglichkeiten reduziert. Es konnte hier gezeigt werden, dass im Gegensatz zu früheren Annahmen *AtPhyB* den Chromophoren Biliverdin, eine Vorstufe des Phytochromobilin, verwenden kann. Die lichtgesteuerte Protein-Protein Interaktion ist unabhängig vom eingebauten Chromophor. Aufgrund der spektralen Eigenschaften des *AtPhyB* mit Biliverdin wurde auf einen neuartigen Bindemechanismus für Biliverdin in pflanzlichen Phytochromen geschlossen. Basierend auf diesem Bindemechanismus ist eine modifizierte Biliverdin-bindende *AtPhyB* Variante erstellt worden, die einen erhöhten Einbaugrad mit Biliverdin zeigt. Die neue Variante könnte somit das Anwendungsmöglichkeiten von *AtPhyB* in anderen Organismen beflügeln.

Neben den pflanzlichen Phytochromen wurde auch das bakterielle Phytochrom aus *Deinococcus radiodurans* (*DrBphP*) untersucht, das als Paradigma für die Strukturbiologie und Signalweiterleitung von Phytochromen fungiert. Bakterielle Phytochrome agieren in der Regel als Histidinkinasen, jedoch wurde für *DrBphP* nie eine solche Funktion festgestellt. In einem kollaborativen Projekt wurde dieses offenstehende Rätsel gelöst und gezeigt, dass *DrBphP* als rotlichtaktivierte Phosphatase agiert. Die innerhalb dieser Arbeit analysierte Interaktion von *DrBphP* mit seinem Antwortregulator unterstützt diese Entdeckung.

Zusammengefasst lässt sich sagen, dass diese Doktorarbeit neue Einblicke in Phytochrome und deren Interaktion mit Partnerproteinen gewährt. Die hierin erbrachten Erkenntnisse werden es ermöglichen die quantitative Untersuchung und Analyse der Signalweiterleitung in Pflanzen besser zu verstehen. Des Weiteren eröffnen sie die Möglichkeiten bereits bestehende lichtabhängige Interaktionssysteme zu optimieren und neue Einsatzgebiete für pflanzliche Phytochrome zu erschließen. Alternative lichtgesteuerte Interaktionssysteme und eine rotlichtaktivierbare Phosphatase stehen außerdem dazu bereit Einsatz in der Optogenetik und Biotechnologie zu finden.

Summary

The detection of light conditions is essential for many organisms, as it allows them to adapt to the environment. For this purpose, organisms possess sensory photoreceptors that respond to different light qualities. Photoreceptors consist of a sensor module, which contains the chromophore for light sensing, and an effector module, which mediates biological function. Owing to the sensitivity, reversibility and spatiotemporal precision of the responses controlled by photoreceptors, they are powerful tools for the control of cellular processes by light, an approach called optogenetics. As one photoreceptor class, phytochromes sense red to far-red light and can be bimodally toggled between two signaling states. Since light of these colors exhibits deep tissue penetration, phytochromes are of considerable interest for optogenetic application.

This thesis studies different aspects of phytochrome systems, with the main focus on light-controlled protein-protein interaction of plant phytochromes. The interaction of the *Arabidopsis thaliana* phytochrome B (PhyB) and its interacting factors (PIFs) is arguably the most prominent red and far-red light-controlled protein-protein interaction. Upon red light illumination, *AtPhyB* undergoes conformational changes that allow interaction with PIFs. The interaction is lost, if the system is kept in dark for a prolonged time or illuminated with far-red light. Although widely used, only sparse and qualitative data are available on the molecular interaction of these proteins. Previous studies identified the photosensory core module of *AtPhyB* and the APB (active phytochrome B binding) motif of PIFs as sufficient for binding. However, quantitative information on interaction strength and dynamics is missing. To address this deficit, several biophysical and fluorescence-spectroscopic methods were established and applied to the interaction of natural and modified PIF proteins with *AtPhyB* under red and far-red light. The findings precisely delineate the APB motif, provide dissociation constants for the interaction, and reveal that the light dependence of the interaction is encoded in the association kinetics.

A. thaliana also possesses several other phytochromes, but little is known about their potential interaction partners. Within a collaborative project, a protein of unknown biological function, denoted OPA, was found to undergo light-dependent interactions with phytochrome A (PhyA) but not with *AtPhyB*. In this thesis, the light-dependent interaction of *AtPhyA* with OPA and a shortened OPA variant is quantitatively analyzed.

To mediate the above and related responses, plant phytochromes utilize the plant-specific chromophore phytochromobilin, which however is generally absent in other organisms.

Therefore, deployment outside of plants mandates the supplementation of suitable chromophores, thus limiting the application scope. This work demonstrates that contrary to the prevalent view in the field, *AtPhyB* can utilize biliverdin, a precursor of the plant-specific chromophore. The light-controlled interaction with PIFs is independent of the incorporated chromophore. Based on spectral analysis, a novel mechanism for biliverdin binding in plant phytochromes was elucidated. On the grounds of this mechanism, a modified biliverdin-binding *AtPhyB* variant was designed which enhanced biliverdin incorporation and thereby, stands to spur the application of *AtPhyB* in other organisms.

This thesis also deals with the bacterial phytochrome of *Deinococcus radiodurans* (*DrBphP*) that serves as a paradigm in structural biology and signal transduction. Bacterial phytochromes generally act as histidine kinases, but previously no catalytic activity was observed for *DrBphP*, although it exhibits pronounced structural responses to light. Within a collaborative project, this long-standing conundrum was resolved by revealing that *DrBphP* acts as red-light-activated phosphatase. The analysis of the interaction that *DrBphP* undergoes with its partner protein, achieved within this thesis, provide support for these findings.

Taken together, this thesis provides unprecedented insight into phytochromes and the interactions they enter with partner proteins. These findings will facilitate the quantitative interrogation and analysis of signal transduction in plants. Moreover, they enable the construction of optimized, light-dependent interaction systems and thereby unlock additional applications for plant phytochromes. Alternative light-controlled interaction systems and a red-light-activated phosphatase stand ready for implementation in optogenetics and biotechnology.

1 Einleitung

Die Absorption von sichtbarem Licht, elektromagnetischer Strahlung mit einer Wellenlänge im Bereich von nahem Ultraviolett (UV, 380 nm) bis hin zu nahem Infrarot (IR, 780 nm), durch Proteinkomplexsysteme erfüllt in der Natur drei entscheidende Aufgaben: Energiegenerierung, Fluoreszenzerzeugung und Adaption des Organismus an seine Umgebung (Gilbert 2012; Presti und Delbruck 1978; Shimomura et al. 1962). Die Photosynthese, die von Pflanzen, Algen und einigen Bakterien betrieben wird, stellt hierbei die Grundlage aller Energieproduktion auf unserem Planeten dar (Arnon 1959). Über die natürliche Funktion von Fluoreszenz ist indes wenig bekannt. Es kann lediglich spekuliert werden, dass Fluoreszenz zur Kommunikation zwischen Organismen genutzt wird (Michiels et al. 2008). Die Wahrnehmung von Licht und daraus resultierende Vermittlung von biologischen Antworten wie Phototaxis (Jékely 2009), Phototropismus (Whippo und Hangarter 2006) und dem Sehprozess bei Tieren (Gehring 2005) wird durch sensorische Photorezeptoren vermittelt.

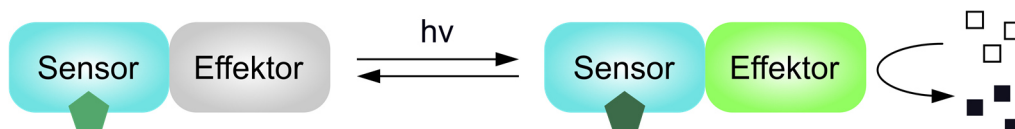


Abbildung 1: Schematische Darstellung des allgemeinen Aufbaus von Photorezeptoren. Das N-terminale Sensormodul ist meist mit einem C-terminalen Effektor verbunden. Der Chromophor für die Wahrnehmung der Lichtbedingungen ist im Sensor enthalten. Nach Absorption von Photonen einer spezifischen Wellenlänge erfolgen strukturelle bzw. dynamische Änderungen, die an den Effektor weitergegeben werden und zu dessen Aktivierung führen (Möglich und Nack 2017)

Photorezeptoren können im Aufbau vereinfacht als zwei funktionelle Module dargestellt werden (Möglich und Moffat 2010; Ziegler und Möglich 2015), dem photosensorischen Modul (Sensor bzw. PSM) und dem Effektormodul (Effektor) (Abbildung 1). Der meist N-terminale Sensor detektiert einfallendes Licht und leitet die erhaltenen Informationen an den meist C-terminal, gekoppelten Effektor weiter (Ziegler und Möglich 2015). In einigen wenigen Ausnahmen ist es jedoch möglich, dass die Domänenabfolge sich vom konventionellen Aufbau unterscheidet und somit der N-terminale Effektor gefolgt wird von einem C-terminalen Sensor (Weber et al. 2019; Losi und Gärtner 2008). Die Wahrnehmung des Lichts erfolgt mit Hilfe eines organischen Chromophors, der in einer Proteindomäne des Sensors gebunden ist. Im dunkeladaptierten Zustand absorbiert der Chromophor Licht spezifischer Wellenlängen und durchläuft anschließend einen Photozyklus dessen Resultat die Bildung des (meta)stabilen Signalzustands ist (Kottke et al. 2018). Der Photorezeptor erfährt in Folge dessen strukturelle

Änderungen, die vom Sensor an den Effektor weitergegeben werden, wodurch der Effektor entweder seine biologische Funktion vermittelt (Christie et al. 1998; Huala et al. 1997) oder in der Ausübung dieser inhibiert wird (Karniol und Vierstra 2003). Durch thermische Reversion kehrt dieser (meta)stabile Zustand über die Zeit oder in einigen Fällen auch gezielt durch Bestrahlung mit Licht einer anderen Wellenlänge zurück in seinen Ausgangszustand, was als Photochromie bezeichnet wird (Rockwell und Lagarias 2010). Die Wellenlängen, die von einem Photorezeptor wahrgenommen werden können, sind dabei stark verknüpft mit dem entsprechenden Chromophoren. Die Einordnung von Photorezeptoren erfolgt in der Regel anhand ihres Chromophoren, Photochemie, Proteinfamilien und taxonomischer Herkunft (Abbildung 2) (Porter 2016; Ziegler und Möglich 2015).

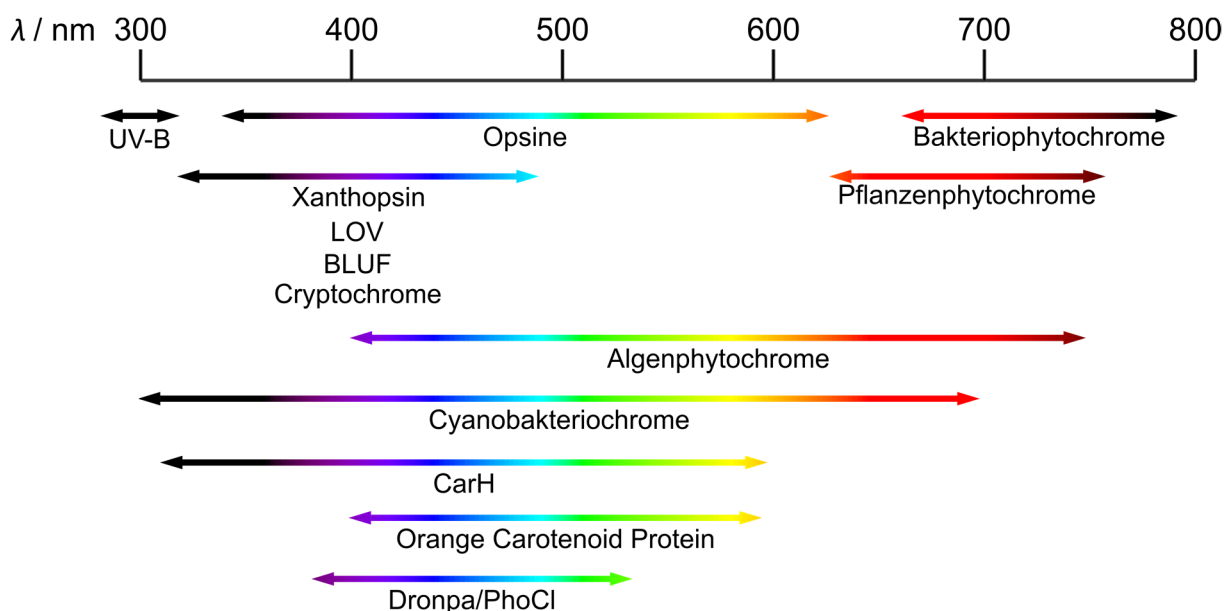


Abbildung 2: Photorezeptoren können anhand ihrer Chromophoren, Photochemie, Proteinfamilien und taxonomischer Herkunft eingeordnet werden (Porter 2016; Ziegler und Möglich 2015).

Während viele Organismen meist nur über einige wenige Klassen von Photorezeptoren verfügen (Fiedler et al. 2005; Davis et al. 1999), haben sich in Pflanzen vermutlich aufgrund ihrer sessilen Lebensweise und der damit verbundenen Notwendigkeit der präzisen Wahrnehmung ihrer Umgebung eine Vielzahl verschiedener Photorezeptorfamilien entwickelt (Weiler 2003; Wada et al. 2005; Möglich et al. 2010). Die UV-B Photorezeptoren, zu denen UVR8 aus *Arabidopsis thaliana* zählt, detektieren Licht im nahen UV Bereich (280 – 315 nm) und verwenden Tryptophane zur Absorption von Photonen (Heijde und Ulm 2012; Wu et al. 2015).

Für die Wahrnehmung von blauem Licht haben sich spezifische Rezeptorgruppen entwickelt, die größtenteils Flavine als Chromophoren beinhalten (Losi et al. 2018). *Light-Oxygen-Voltage-sensing* (LOV) Photorezeptoren wurden zunächst in Pflanzen als Mediator für den Phototropismus entdeckt (Huala et al. 1997; Christie et al. 1998), jedoch stellte sich bereits sehr bald heraus, dass diese Proteinfamilie auch in Bakterien, Pilzen und Cyanobakterien vorhanden ist (Losi und Gärtner 2012). Eine weitere Klasse der Blaulichtrezeptoren sind Cryptochrome, die in Pflanzen das Wachstum (Zlotorynski 2016) und in Tieren den zirkadianen Rhythmus kontrollieren (Yu et al. 2010). *Sensors of Blue Light Using Flavin adenine dinucleotide* (BLUF) werden primär in Proteobakterien gefunden und regulieren dort oftmals den zyklischen Nukleotidmetabolismus (Losi et al. 2018). Die letzte Klasse der Blaulichtrezeptoren sind Xanthopsine, zu denen das *photoactive yellow protein* (PYP) zählt (Borucki 2006). Im Gegensatz zu anderen Blaulichtrezeptoren verwenden Xanthopsine die 4-Hydroxymethylsäure als Chromophor und sind vermutlich an der negativen Phototaxis in halophilen Bakterien beteiligt (Sprenger et al. 1993).

Der Bereich des für den Menschen sichtbaren Lichts (380 – 780 nm) wird von Opsinen mit Hilfe von 11-*cis*-Retinal detektiert (Müller et al. 2019). Außer in Säugertieren findet man Opsine in Mikroorganismen und Algen, in denen sie z. B. als lichtabhängige Ionenkanäle fungieren (Nagel et al. 2002; Nagel et al. 2003; Gushchin und Gordeliy 2018). Neben den Opsinen detektieren unter anderem erst kürzlich entdeckte Rezeptorklassen eine große Bandbreite des sichtbaren Lichts. Die CarH Proteine sind UV, Blau- und Grünlicht Sensoren, die Vitamin B₁₂ in Form von 5'-Deoxyadenosylcobalamin als Kofaktor verwenden und als Transkriptionsfaktoren in Bakterien agieren (Padmanabhan et al. 2017; Padmanabhan et al. 2019). Einige Cyanobakterien verwenden zum Schutz vor zu hohen Lichtintensitäten *orange carotenoid proteins*, eine Photorezeptorfamilie die Carotin als Chromophor verwendet und mit Phycobilisomen der Cyanobakterien interagieren (Muzzopappa und Kirilovsky 2020). Zu den weiteren erst kürzlich näher charakterisierten Klassen zählen die vom grün fluoreszierenden Protein (*green fluorescent protein*, GFP) abgeleiteten Photorezeptoren wie beispielsweise Dronpa, welches durch die Absorption von UV- und Blaulicht unterschiedliche Oligomersierungszustände eingeht (Jöhr et al. 2019), oder das photospaltbare Protein (*photocleavable protein*, PhoCl) bei dem es durch violettes Licht zu einem spezifischen Bruch im Proteinrückgrat kommt (Zhang et al. 2017).

Eine besonders vielseitige Familie der Photorezeptoren sind die Phytochrome. Ursprünglich als Rot- und Fernrotlicht (600 nm – 780 nm) detektierende Rezeptoren in Pflanzen (PhyA-E)

entdeckt (Butler et al. 1959), konnten Phytochrome mittlerweile auch in Bakterien (BphP), Pilzen (Fph), Algen (Dph) und Cyanobakterien (Cph) nachgewiesen werden (Karniol et al. 2005). In Mikroorganismen erfüllen Phytochrome eine wichtige Rolle bei der Phototaxis (Narikawa et al. 2013; Jékely 2009) und in Pflanzen regulieren sie eine Vielzahl von Prozessen, darunter Samenkeimung und Schattenvermeidung (Kreslavski et al. 2018). Phytochrome und phytochrom-ähnliche Cyanobakteriochrome (CBCRs) verwenden lineare Tetrapyrrole, sogenannte Biline, als Chromophore und können abhängig von ihrer spezifischen Aminosäuresequenz ein breites Spektrum des sichtbaren Lichts detektieren (Abbildung 2).

Im Folgenden wird auf die Charakteristika von Phytochromen, dem Hauptthema dieser Arbeit, eingegangen.

1.1 Phytochrome

Beginnend mit Experimenten bei denen vom tageslichtabhängigen Verhalten von Pflanzen auf hierfür verantwortliche Bestandteile geschlussfolgert wurde (Garner und Allard 1920), über den ersten Nachweis von Phytochromen (Butler et al. 1959), bis hin zur molekularen Charakterisierung der heutigen Zeit (Burgie et al. 2014a; Takala et al. 2014) umfasst die Geschichte der Phytochromforschung mittlerweile einen Zeitraum von ungefähr 100 Jahren (Sage 1992). Phytochrome werden dabei als bilinbindende Proteine zusammengefasst, die abhängig von der Beleuchtung zwei stabile Zustände einnehmen können (Rockwell und Lagarias 2010).

Das Sensormodul von Phytochromen setzt sich in der Regel aus einer PAS (Per-ARNT-SIM) (Möglich et al. 2009b), einer GAF (*cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA*) (Aravind und Ponting 1997) und einer PHY (*phytochrome-specific*) Domäne zusammen (Yang et al. 2008; Rockwell und Lagarias 2010; Essen et al. 2008). Der Chromophor ist dabei in der GAF Domäne eingebettet und kovalent über ein Cystein in PAS oder GAF Domäne mit dem Protein verbunden (Abbildung 3). Bei den CBCRs genügt indes lediglich die GAF Domäne als Sensor und ist für die Ausbildung beider Signalzustände ausreichend (Ikeuchi und Ishizuka 2008).

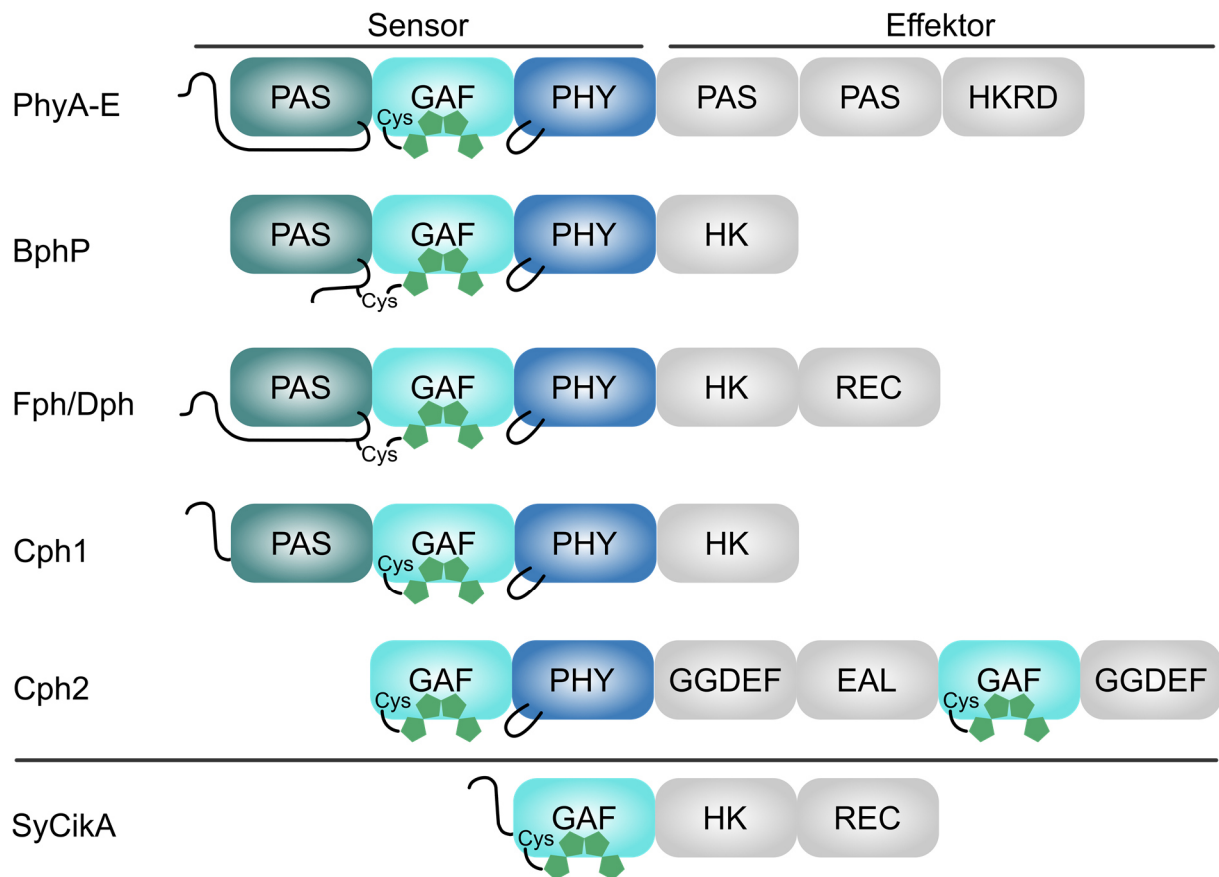


Abbildung 3: Schematischer Aufbau der Phytochrome und Cyanobakteriochrome. Phytochrome können in Sensor und Effektor gegliedert werden. Der Sensor setzt sich in der Regel aus PAS, GAF und PHY Domäne zusammen wobei die PAS-Domäne bei einigen cyanobakteriellen Phytochromen fehlen kann. Der Bilinchromophor (grün) ist in der GAF Domäne eingebettet und kovalent über ein Cystein (Cys), welches sich entweder in der GAF oder der PAS Domäne befindet, mit dem Protein verbunden. In Phytochromen aus Pflanzen, Algen, Pilzen und teilweise auch aus Cyanobakterien befindet sich vor der ersten PAS Domäne noch eine N-terminale Erweiterung. Bei der Effektordomäne von Phytochromen handelt es sich in der Regel um eine Histidinkinase (HK). Bei pflanzlichen Phytochromen folgen der PHY Domäne jedoch zunächst zwei zusätzliche PAS Domänen und eine Histidinkinase-ähnliche Domäne (*histidine kinase related domain*, HKRD). In Phytochromen aus Algen und Pilzen ist häufig eine C-terminale *response-regulator receiver* Domäne (REC) konserviert. Einige cyanobakterielle Phytochrome verwenden außerdem GGDEF Domänen als Effektor. Cyanobakteriochrome, z.B. SyCikA, sind phytochrom-ähnliche Photosensoren, die lediglich GAF Domänen als Sensor verwenden und unterschiedliche Effektordomänen besitzen können (Rockwell und Lagarias 2010; Ikeuchi und Ishizuka 2008).

Phytochrome verwenden lineare Tetrapyrrole als Chromophore, die im Zuge des katabolen Porphyrinstoffwechsels gebildet werden (Spudich und Briggs 2005). Die am häufigsten verwendeten Chromophore sind Biliverdin (BV) in bakteriellen Phytochromen und dessen reduzierte Stoffwechselprodukte Phytochromobilin (PΦB) in pflanzlichen Phytochromen bzw. Phycocyanobilin (PCB) in cyanobakteriellen Phytochromen und CBCRs, jedoch werden vereinzelt auch Phycoviolobilin (PVB) oder Bilirubin genutzt (Rockwell und Lagarias 2010).

Unabhängig von der genauen Art des Bilins im Phytochrom durchlaufen diese eine reversible Z zu E Isomerisierung der Doppelbindung zwischen dem C15 und dem C16 Atom nach Rotlichtbestrahlung was eine Drehung des D-Ring bewirkt (Abbildung 4). Die resultierende E-Konformation kehrt im Dunkeln oder nach Fernrotlichtbestrahlung zurück in die Z-Konformation. Der Zustand des Chromophors legt somit fest, ob das Phytochrom Rotlicht oder Fernrotlicht absorbiert und damit ob es sich im Pr oder Pfr Zustand befindet. Die biologische Aktivität von Phytochromen ist stark mit entsprechenden Beleuchtungszuständen verknüpft. Beim Effektormodul handelt es sich bei bakteriellen Phytochromen in der Regel um Histidinkinasen (HK). Abhängig vom Ursprungsorganismus können auch andere Domänen als Effektor fungieren (Abbildung 3). Alternativ kann die biologische Funktion durch lichtabhängige Protein-Protein Interaktion vermittelt wird (Pham et al. 2018).

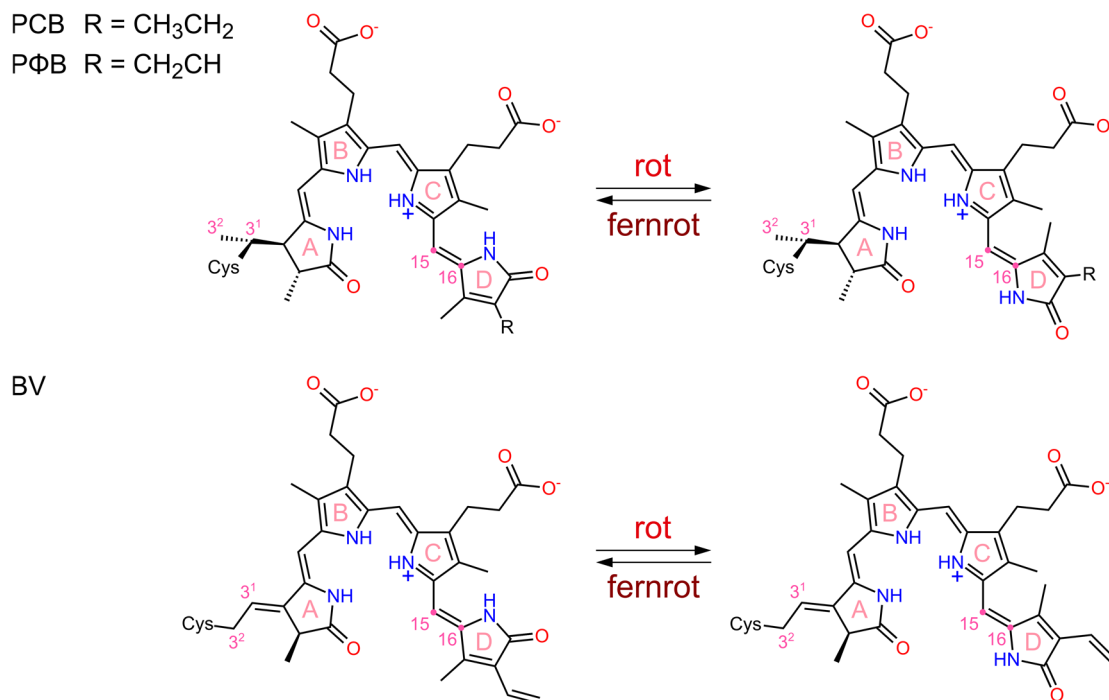


Abbildung 4: Darstellung der kovalenten Bindung und der Photoisomerisierung der Chromophore in Phytochromen. Die Bindung des Chromophoren zum Phytochrom entsteht durch Reaktion der Vinyl Gruppe des A-Ring mit einem Cystein des Proteins. Phycocyanobilin (PCB) und Phytochromobilin (PΦB) binden jeweils über das C3¹ Atom an ein Cystein in der GAF Domäne. PCB und PΦB unterscheiden sich lediglich durch eine Ethyl- bzw. Vinyl Gruppe am D-Ring. Biliverdin (BV), welches in bakteriellen Phytochromen zum Einsatz kommt, bildet eine kovalente Bindung mit einem Cystein der PAS-Domäne über das C3² Atom aus. Nach Rotlichtabsorption erfolgt eine Z zu E Isomerisierung der Doppelbindung zwischen C15 und C16, die im Dunkeln oder nach Fernrotlichtabsorption in den Ausgangszustand zurückkehrt (Mroginski et al. 2007).

Im Folgenden soll auf die spezifischen Details der wichtigsten Phytochromfamilien eingegangen werden.

1.1.1 Struktur von Phytochromen

Phytochrome weisen in ihrem Sensor eine Domänenarchitektur bestehend aus jeweils einer PAS, GAF und PHY Domäne auf. Vor allem bei den Cyanobakterien lassen sich jedoch Ausnahmen von dieser Regel finden bei denen keine N-terminale PAS Domäne vorhanden ist (Anders et al. 2013; Rockwell und Lagarias 2010). Der Sensor bei den CBCRs besteht hingegen lediglich aus einer GAF Domäne und ist vollkommen ausreichend für Photochromie (Ikeuchi und Ishizuka 2008).

Soweit strukturell untersucht, liegen Phytochrome in der Regel als parallele Homodimere vor (Burgie et al. 2014a; Burgie et al. 2014b). Die Fähigkeit Dimere zu bilden wird dabei nicht nur vom Sensor vermittelt, sondern in Teilen auch vom Effektor was in pflanzlichen Phytochromen durch die Entfernung des C-terminalen Effektors gezeigt wurde (Burgie et al. 2017). Betrachtet man den klassischen Aufbau des Sensormoduls innerhalb unterschiedlicher Familien, so zeigen sich aber auch hier Unterschiede zwischen den einzelnen Familien. In den Phytochromen aus Cyanobakterien, Pilzen, Algen und höheren Pflanzen befinden sich vor der ersten PAS Domäne noch weitere Aminosäuren, die N-terminale Erweiterung (*N-terminal extension*, NTE). Die Länge der NTE ist dabei in der Regel vom Herkunftsorganismus abhängig. Besonders in pflanzlichen Phytochromen ist die Rolle der NTE bereits intensiver erforscht und es konnte gezeigt werden, dass dieser einen Einfluss auf die Stabilität des Pfr Zustandes hat (Velázquez Escobar et al. 2017a). Des Weiteren beinhaltet die NTE in pflanzlichen Phytochromen Phosphorylierungsstellen, die sowohl die thermale Reversion als auch die biologische Funktion beeinflusst (Medzihradszky et al. 2013; Nito et al. 2013; Viczián et al. 2020). Am Übergang der NTE in die PAS Domäne befindet sich ein weiteres charakteristisches Element der Phytochrome, die Knotenstruktur, bei der eine Schleife der GAF Domäne einen Bereich der PAS Domäne umspannt. Im Phytochrom B aus *Arabidopsis thaliana* (*AtPhyB*) scheint diese Region zentralen Einfluss auf die Interaktion mit Partnerproteinen zu haben (Kikis et al. 2009). Die genaue Bindestelle der Partnerproteine an Phytochromen ist jedoch aktuell noch unbekannt. Die PAS Domäne besitzt die räumliche Struktur eines fünfsträngigen antiparallelen β -Faltblatts umgeben von drei α -Helices und befindet sich in engem Kontakt zur GAF Domäne, die aus einem sechssträngigen antiparallelen β -Faltblatts und fünf α -Helices besteht (Wagner et al. 2005). Die GAF Domäne beinhaltet den organischen Chromophor in einer Bindetasche, die aus dem β -Faltblatt und zwei der α -Helices aufgebaut ist (Burgie et al. 2014a). Zusätzlich zu den Aminosäuren der GAF Domäne sind auch Aminosäuren von PAS- und PHY-Domäne für einen korrekten Einbau des Bilins notwendig (Abbildung 5). Diese Aminosäuren sind

hochkonserviert zwischen Phytochromen aus unterschiedlichen Familien (Wagner et al. 2008; Burgie und Vierstra 2014; Burgie et al. 2014a).

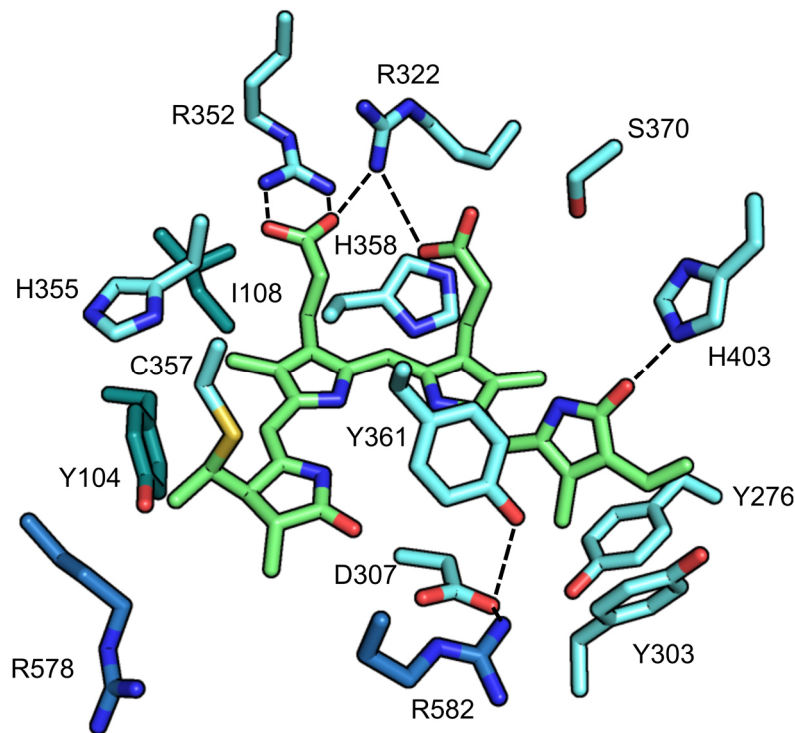


Abbildung 5: PΦB Chromophor und Aminosäuren der Bindetasche von *AtPhyB* (PDB-Eintrag 4our). Der PΦB Chromophor ist grün hervorgehoben. Die Aminosäuren von PAS-, GAF- und PHY-Domäne sind blaugrün, cyan und dunkelblau eingefärbt. Wasserstoffbrückenbindungen sind in gestrichelten Linien eingezeichnet (Burgie et al. 2014a).

Für die Ausbildung der kovalenten Bindung zum Chromophoren wird in bakteriellen Phytochromen in der Regel ein Cystein in der PAS Domäne verwendet (Takala et al. 2014). Bei pflanzlichen und cyanobakteriellen Phytochromen kommt ein Cystein in der GAF Domäne zum Einsatz (Abbildung 5). Die Absorption von Rotlicht führt zur charakteristischen Z zu E Isomerisierung des Chromophoren, die eine Drehung des D-Rings zur Folge hat. Die Änderung wird von umgebenden Aminosäuren wahrgenommen wodurch ein Strukturänderungssignal an die PHY Domäne weitergegeben wird. Der Abstand der beiden PHY Domänen des Phytochromdimers vergrößert sich infolgedessen (Abbildung 6). Die PHY-Zunge, ein von der PHY Domäne ausgehendes Strukturelement, das direkte Interaktion mit der Chromophorbindetasche der GAF Domäne ausübt, durchlebt eine reversible strukturelle Änderung von β -Schleife im Pr Zustand zu α -Helix im Pfr Zustand. Dies konnte jedoch aktuell nur für bakterielle Phytochrome gezeigt werden (Takala et al. 2014; Takala et al. 2015). Im Fall der Bakteriophytochrome gehen die C-terminalen Helices der PHY Domänen stufenlos in die Dimerisierungs- und Phosphatakzeptor-Histidin (*dimerization and phospho-accepting*

histidine, DHp) Domäne der Histidinkinasen über, wodurch das Signal an den Effektor weitergegeben wird (Möglich 2019). Da für viele Phytochrome jedoch lediglich Strukturen des Sensors ohne den Effektor vorhanden sind, ist der genaue Mechanismus der Signalweiterleitung in anderen Phytochromfamilien noch weitestgehend unbekannt. Es wird allerdings anhand der bereits vorhandenen Strukturen geschlussfolgert, dass die Signalweiterleitung vermutlich primär über *coiled-coil* Helixstrukturen erfolgt (Otero et al. 2016; Gourinchas et al. 2017).

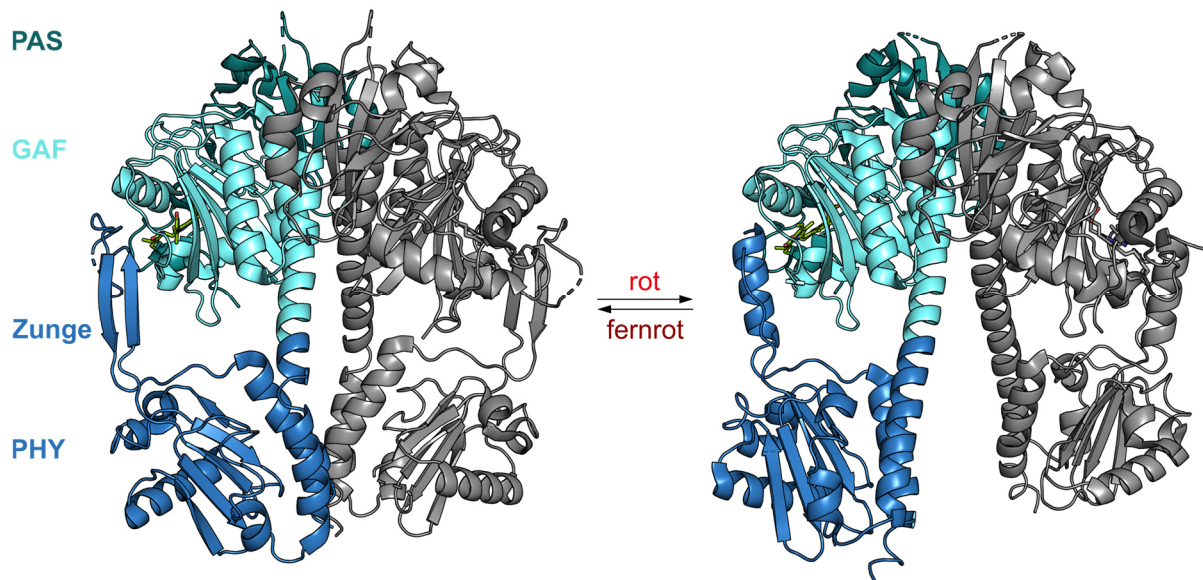


Abbildung 6: Struktur des isolierten Sensormoduls des *Deinococcus radiodurans* Bakteriophytochroms (*DrBphP*) im dunkel- (PDB-Eintrag 4o0p) und lichtadaptierten Zustand (PDB-Eintrag 4o01). Nach Beleuchtung mit Rotlicht kommt es zu strukturellen Änderungen in der Verbindungshelix zwischen GAF und PHY Domäne, wodurch sich die PHY Domänen des parallelen Dimers voneinander wegbewegen. Die Zunge nimmt im dunkeladaptierten Zustand die Struktur eines β -Faltblatts ein, welches nach Rotlicht Bestrahlung zu einer α -Helix umgewandelt wird.

1.1.2 Chromophore und Photozyklus

Biline sind offenkettige lineare Tetrapyrrole, die im Zuge des Porphyrinstoffwechsels aus Metalloporphyrinen, Häm oder Chlorophyll gebildet werden. Aufgrund ihres konjugierten π -Elektronensystems absorbieren Biline Licht im sichtbaren Spektrum wodurch Sie in der Natur für Pigmentierung, Lichtwahrnehmung und Energiegewinnung in Lichtsammelkomplexen Verwendung finden (Frankenberg und Lagarias 2000). Biliverdin (BV) ist das Primärprodukt des Häm-Katabolismus in Tieren und wird vom Enzym Hämoxygenase (HO) gebildet (Abbildung 7) (Liu und Ortiz de Montellano 2000; Mahawar und Shekhawat 2018; Ortiz de Montellano 2000).

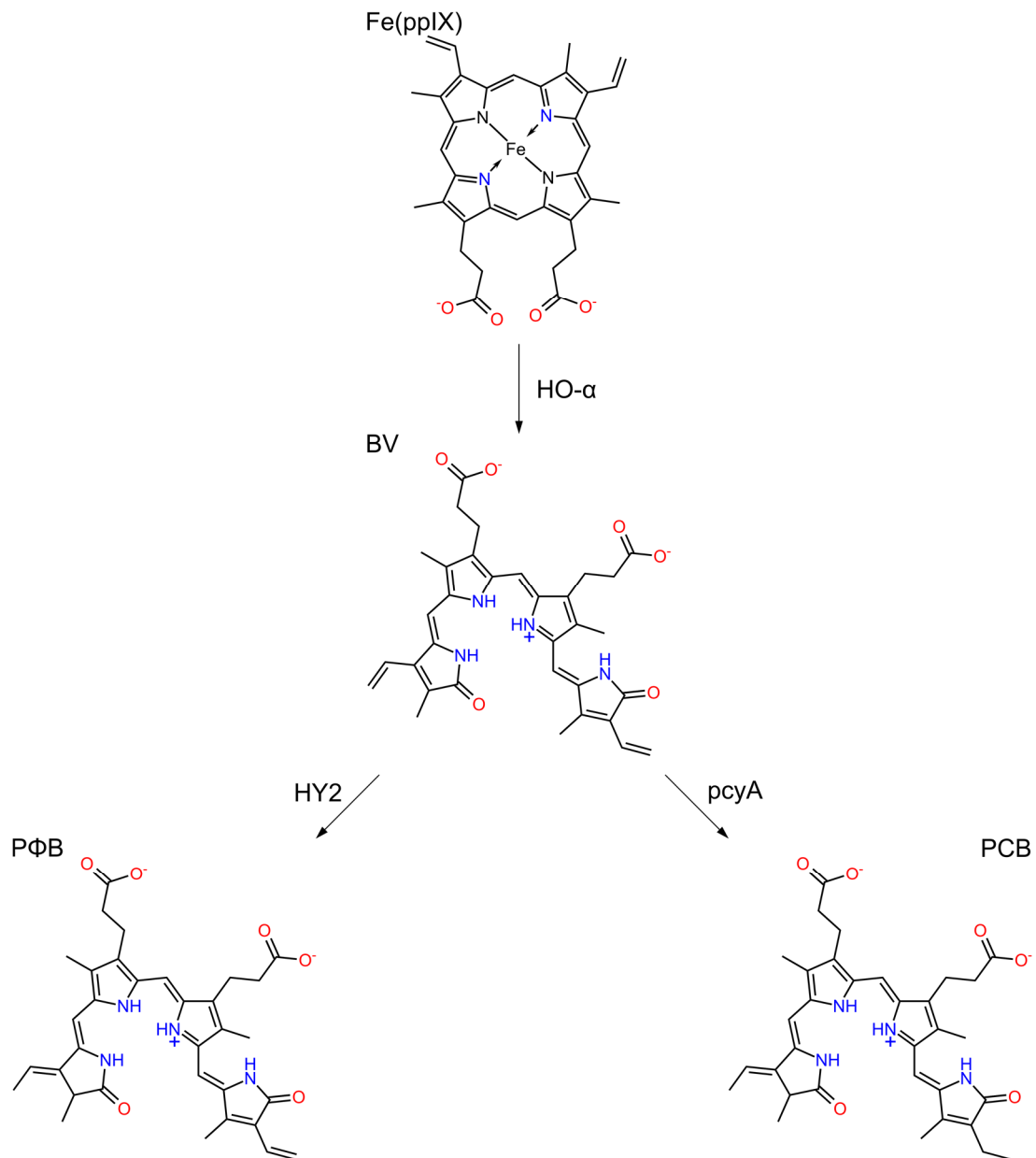


Abbildung 7: Synthese der wichtigsten Biline, die als Chromophore in Phytochromen dienen. Fe-Protoporphyrin IX [Fe(ppIX)] wird von Hämoxygenase- α (HO- α) in Biliverdin IXa (BV) umgewandelt, welches als Chromophor in Bakteriophytochromen zum Einsatz kommt. BV ist die Vorstufe von Phytochromobilin (PΦB) und Phycocyanobilin (PCB), die von den Enzymen Ferredoxin Oxidoreduktase (HY2) bzw. Phycocyanobilin:ferredoxin Oxidoreduktase (pcyA) gebildet werden. PΦB kommt in pflanzlichen Phytochromen zum Einsatz, während PCB als Chromophor von cyanobakteriellen Phytochromen und CBCRs verwendet wird (Frankenberg und Lagarias 2000).

In Tieren wird das Oxidationsmittel für die Spaltung der Methinbrücke von der NADPH-abhängigen Cytochrome P450 Reduktase bereitgestellt, während pflanzliche und cyanobakterielle Hämoxygenasen Ferredoxin-abhängig sind (Frankenberg und Lagarias 2000). Die in Bakteriophytochromen als Chromophor verwendete Isoform ist Biliverdin IXa, welche über ihr C3² Atom kovalent an ein Cystein in der PAS-Domäne des Phytochroms gebunden

wird. BV wird in Tieren abgebaut zu Bilirubin, in Pflanzen zu Phytochromobilin (PΦB) und in Cyanobakterien zu Phycocyanobilin (PCB) (Abbildung 7). PΦB und PCB sind die Chromophore, die in pflanzlichen bzw. cyanobakteriellen Phytochromen zum Einsatz kommen (Rockwell und Lagarias 2010).

Die Ferredoxin Oxidoreduktase HY2 in Pflanzen, auch PΦB Synthase genannt, bildet PΦB durch eine zwei Elektronen Reduktion aus BV (Kohchi 2001; Frankenberg 2001). Auf analoge Weise wird PCB in Cyanobakterien mit Hilfe der Phycocyanobilin:ferredoxin Oxidoreduktase pcyA generiert (Uda et al. 2017). PΦB und PCB unterscheiden sich lediglich anhand einer Ethyl- bzw. Vinyl Gruppe am D-Ring (Abbildung 7). Die Bindung dieser Biline in pflanzlichen und cyanobakteriellen Phytochromen erfolgt zwischen dem C3¹ Atom und einem strukturell konservierten Cystein der GAF-Domäne. Aufgrund der molekularen Ähnlichkeit der Chromophore ist es möglich PΦB in pflanzlichen Phytochromen durch PCB zu substituieren (Mroginski et al. 2011; Kyriakakis et al. 2018). Besonders bei der Anwendung von pflanzlichen und cyanobakteriellen Phytochromen bzw. CBCRs in Bakterien, Säugetierzellkultur und in Modelorganismen ist es daher notwendig, dass die entsprechenden Enzyme für die Herstellung der Chromophoren vorhanden sind (Cornejo et al. 1998; Kyriakakis et al. 2018; Lu et al. 2017; Mukougawa et al. 2006; Uda et al. 2017). Alternativ kann der PCB Chromophor auch extern über das Medium zugeführt werden (Toettcher et al. 2011b; Müller et al. 2013a).

Nach Einbau des Chromophors in das entsprechende Phytochrom zeigt dieses die charakteristischen Pr bzw. Pfr Spektren (Abbildung 8). Der Pr-Zustand ist in der Regel der dunkeladaptierte Zustand, jedoch gibt es einige Phytochrome, Bathyphytochrome, bei denen im Dunkeln der Pfr-Zustand adaptiert wird (Rottwinkel et al. 2010; Salewski et al. 2013; Velázquez Escobar et al. 2017b). Der durch Rotlicht erzeugte Zustand ist in der Regel ein Mischzustand aus den reinen Pr und Pfr Spektren, da beide Zustände Rotlicht absorbieren können (Butler et al. 1964). Der Pr Zustand cyanobakterieller Phytochrome und pflanzlicher Phytochrome besitzt ein Absorptionsmaxima von ca. 655 nm für PCB und ca. 665 nm für PΦB (Lamparter 2004). Bakteriophytochrome mit BV weisen dahingegen ein um ca. 50 nm rotverschobenes Pr-Absorptionsmaxima bei ca. 700 nm auf (Quest und Gärtner 2004; Auldridge und Forest 2011).

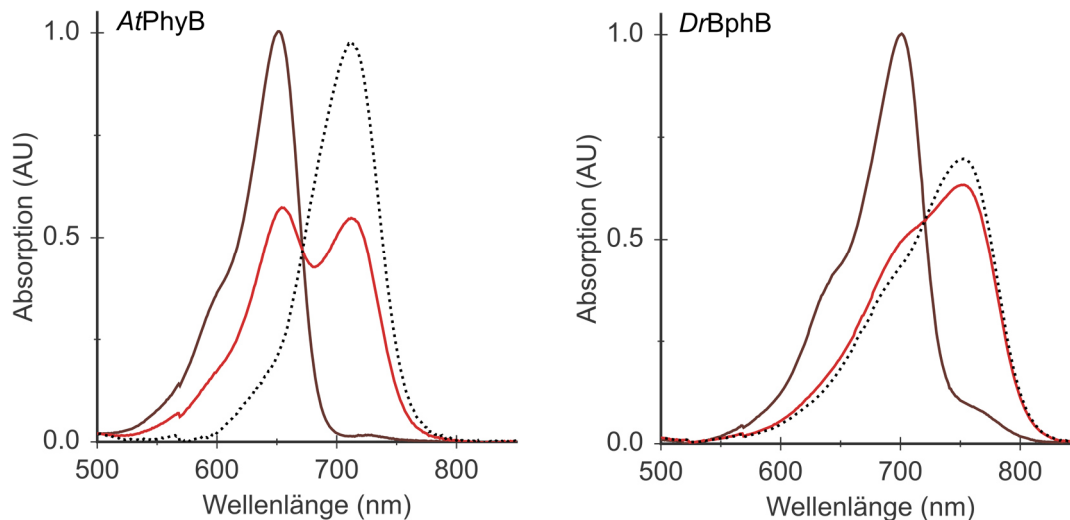


Abbildung 8: Absorptionsspektren des Pflanzenphytochroms aus *Arabidopsis thaliana* (AtPhyB) und des Bakteriophytochroms aus *Deinococcus radiodurans* (DrBphP) nach Rotlicht (rot) und Fernrotlicht (braun) Bestrahlung und das berechnete Spektrum für den reinen Pfr Zustand (gepunktete Linie)

Nach Absorption eines Photons mit Wellenlänge im Rotlichtbereich durchläuft der Chromophor einen charakteristischen Photozyklus bei dem über einige kurzlebige Zwischenstufen zunächst ein (meta)stabiles Produkt, Pfr-Zustand, gebildet wird (Abbildung 9). Die Anzahl der Zwischenstufen variiert dabei abhängig vom Phytochrom (Björling et al. 2016; Uljasz und Vierstra 2011). Innerhalb von Pikosekunden nach der Anregung durch Rotlicht kommt es zur Ausbildung des Lumi-R Zustandes, der nach Mikrosekunden durch thermische Reversion in den Meta-R_a-Zustand übergeht. Deprotonierung führt zunächst zur Ausbildung des meta-R_c-Zustandes, welcher durch Protonierung in den (meta)stabilen Pfr Zustand überführt wird. Bildung und Zerfall des meta-R_c erfolgt im Millisekunden Zeitraum (Uljasz und Vierstra 2011). Der Pfr Zustand kehrt nach einiger Zeit im Dunkeln wieder in den Pr Zustand zurück, wobei die Geschwindigkeit der thermischen Reversion vom jeweiligen Phytochrom (Remberg et al. 1998), dem eingebauten Chromophor (Remberg et al. 1998), der strukturellen Vollständigkeit des Phytochroms (Burgie et al. 2017) und der Beschaffenheit der Aminosäuren in der Chromophorbindetasche (Zhang et al. 2013) abhängig ist. Der Pfr-Zustand kann auch durch Fernrotlichtbestrahlung in den Pr-Zustand überführt werden und durchläuft dabei eine ähnliche Reaktionsfolge wie nach Rotlichtbestrahlung (Abbildung 9). Zunächst wird nach einigen Pikosekunden der Lumi-F-Zustand gebildet, der innerhalb von Nanosekunden in den Meta-F_a-Zustand überführt wird. Der Meta-F_a wird anschließend innerhalb von Mikrosekunden in den Meta-F_b und daraufhin nach einigen Millisekunden in den Pr-Zustand überführt (Uljasz und Vierstra 2011). Der Übergang zwischen Pr und Pfr Zustand ist mit der

Isomerisierung der Doppelbindung zwischen C15=C16 der Bilinchromophore verknüpft, die während der Bildung der Lumi-Zustände erfolgt.

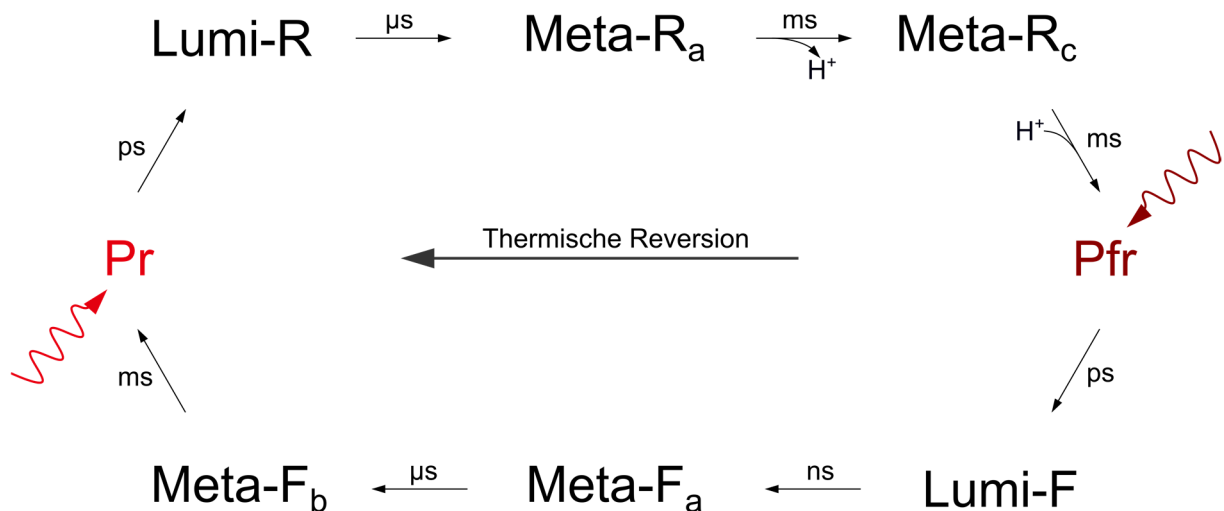


Abbildung 9: Vereinfachter Photozyklus von Phytochromen. Nach Absorption von Rotlicht erfolgt der Übergang vom Pr in den Pfr Zustand über mehrere Zwischenstufen. Zunächst kommt es innerhalb von Pikosekunden, durch Isomerisierung der C15=C16 Doppelbindung, zur Ausbildung des Lumi-R Zustands, welcher nach einigen Mikrosekunden in den Meta-R_a Zustand übergeht. Daraufhin wird der Meta-R_c gebildet und in den Pfr Zustand überführt. Bildung und Zerfall des Meta-R_c geschieht auf der Millisekunden Zeitskala. Der Pfr Zustand kann entweder durch thermische Reversion im Dunkeln oder nach Fernrotlichtbeleuchtung wieder in den Pr Zustand überführt werden. Die einzelnen Prozesse laufen dabei analog zum Pr → Pfr Übergang mit leicht veränderten Zeitkonstanten ab, wobei die gebildeten Zwischenprodukte jedoch Lumi-F, Meta-F_a und Meta-F_b genannt werden (Ulijasz und Vierstra 2011).

Neben den klassischen Rot- und Fernrotlicht absorbierenden Phytochromen haben sich in der Familie der Cyanobakterien eine Vielzahl von phytochrom-ähnlichen CBCRs entwickelt, deren Farbsensitivität nicht nur auf das rote Spektrum des sichtbaren Lichts begrenzt ist (Rockwell und Lagarias 2010). Die resultierenden Photozyklen in CBCRs sind divers und der dunkeladaptierten Zustand kann sich, z. B. innerhalb der Grün- und Rotlicht absorbierenden CBCRs, unterscheiden (Hirose et al. 2010; Narikawa et al. 2008; Rockwell et al. 2012). Eine Blauverschiebung der Absorptionsspektren bis hin in den nahen UV Bereich wird durch ein weiteres Cystein bewirkt, das eine zusätzliche kovalente Bindung zum C¹⁰ Atom des Bilin Chromophoren ausbildet (Rockwell et al. 2011; Rockwell et al. 2008). Der Grund für die Entwicklung dieser phytochrom-ähnlichen Photorezeptoren ist vermutlich darauf zurückzuführen, dass Cyanobakterien für ihr Überleben sowohl auf die Energieproduktion durch Lichtabsorption angewiesen sind, aber gleichzeitig versuchen schädliche Lichtbedingungen zu vermeiden (Song et al. 2011; Narikawa et al. 2013).

Kürzlich wurden aber auch einige Algenphytochrome identifiziert deren Absorptionsspektren ebenfalls deutliche Abweichungen vom klassischen Rot und Fernrotlicht Verhalten aufweisen (Rockwell et al. 2014; Duanmu et al. 2014). Auch hier scheint eine deutliche Blauverschiebung durch ein weiteres Cystein bewirkt zu werden (Duanmu et al. 2014).

1.1.3 Histidinkinasen als Effektor in bakteriellen Phytochromen

Die biologische Aufgabe von Phytochromen ist die Wahrnehmung von Lichtbedingungen und Umwandlung des Lichtsignals in eine biologische Antwort. Bakterielle Phytochrome bewirken ihre biologische Aktivität meist als Bestandteil von Zweikomponentensystemen mit Hilfe ihrer Histidinkinase-Effektordomäne indem sie den Phosphorylierungszustand von Antwortregulatoren beeinflussen (Bhoo et al. 2001; Möglich 2019). Der Großteil unseres Wissens bezüglich der biologischen Aufgabe von bakteriellen Phytochromen, abseits der Cyanobakterien, bezieht sich auf Bakterienarten, die Interaktionen mit Pflanzen eingehen (Beattie et al. 2018). Es konnte gezeigt werden, dass Phytochrome in diesen Bakterienarten lichtabhängig unter anderem die Synthese des Bakteriochlorophylls kontrollieren (Giraud et al. 2002), Konjugation (DNA Transfer) zwischen Agrobakterien regulieren (Bai et al. 2016; Xue et al. 2019) oder über Genexpression, Beweglichkeit und Virulenz beeinflussen (McGrane und Beattie 2017; Bonomi et al. 2016; Wu et al. 2013).

Aufgrund ihrer Löslichkeit und der einfachen Zustandskontrolle mittels Licht haben sich Photorezeptor-Histidinkinasen in der Vergangenheit als optimale Plattform zu Untersuchung der Histidinkinasefamilie bewährt (Diensthuber et al. 2013; Jacob-Dubuisson et al. 2018; Möglich 2019). Histidinkinasen, in der Regel Homodimere (Rivera-Cancel et al. 2014), agieren abhängig von ihrem Signalzustand entweder als Kinasen oder Phosphatasen. Als erster Reaktionsschritt erfolgt die Autophosphorylierung der Histidinkinase. Dabei wird die γ -Phosphatgruppe von einem ATP auf das katalytisch aktive Histidin übertragen (Abbildung 10a). Die phosphorylierte Histidinkinase kann daraufhin die Phosphatgruppe auf einen spezifischen Asparaginsäurerest im Antwortregulator übertragen (Abbildung 10b). Die Dephosphorylierung des Antwortregulators erfolgt durch die Hydrolyse der Phosphorsäureesterbindung unter Verbrauch eines Wassermoleküls (Abbildung 10c). Für alle Reaktionsschritte werden bivalente Metallionen benötigt, in der Regel Mg^{2+} (Stock et al. 2000).

Obwohl es eine Vielzahl von Kristallstrukturen des Sensormoduls bakterieller Phytochrome gibt (Burgie et al. 2016; Takala et al. 2014; Yang et al. 2008), fehlt bis dato noch eine vollständige Struktur mit dem Histidinkinase-Effektor, die die Verbindung zwischen PHY

Domäne und DHp Domäne über die *coiled-coil* Helices zeigt. Unabhängig von der Art der Histidinkinase gibt es jedoch eine sehr hohe Verwandtschaft zwischen den Histidinkinasen. Die Konservierung zeigt sich in der Signalweiterleitung via *coiled-coil* Helices zum Effektor, der Ähnlichkeit der DHp Domäne als auch der katalytischen (*catalytic*, CA) Domäne im Effektor und schlussendlich durch die Kinase- bzw. Phosphataseaktivität (Möglich 2019). Aus diesem Grund können sehr gut charakterisierte Histidinkinasen wie das DesK aus *Bacillus subtilis*, das HK853 aus *Thermotoga maritima* oder das künstlich erzeugte, lichtabhängige YF1 als Model für Histidinkinase-Antwortregulator Zweikomponentensysteme betrachtet und von diesen auf andere Histidinkinasesysteme geschlussfolgert werden (Trajtenberg et al. 2016; Casino et al. 2009; Casino et al. 2014; Diensthuber et al. 2013; Möglich et al. 2009a).

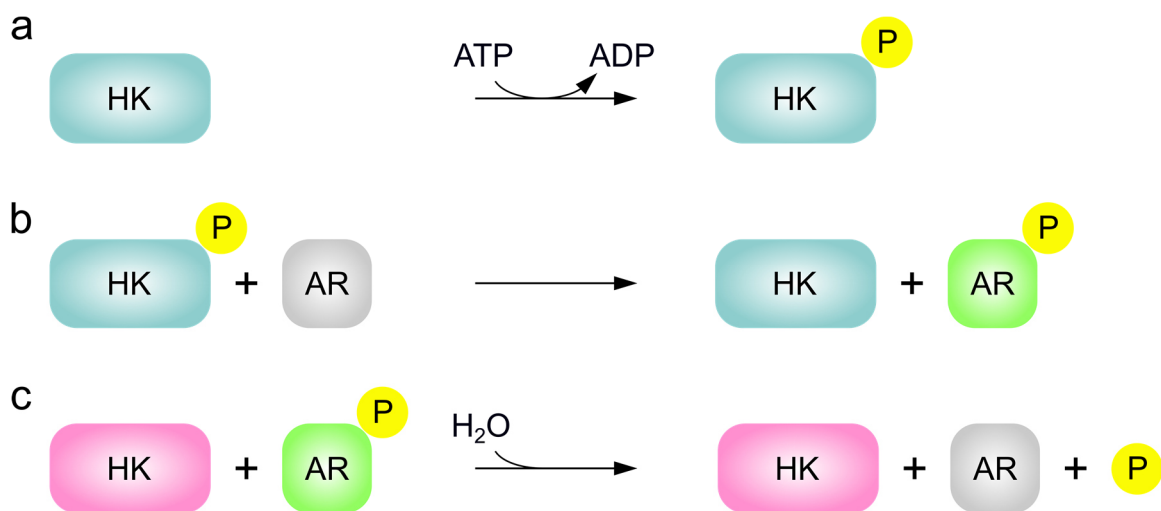


Abbildung 10: Schematische Darstellung der drei von Histidinkinasen (HK) katalysierten Reaktion. (a) Unter Verbrauch eines ATP Moleküls wird während der Autophosphorylierungsreaktion eine Phosphatgruppe auf die Histidinkinase übertragen. (b) Die Phosphatgruppe wird anschließend von der Histidinkinase auf den Antwortregulator (AR) transferiert. (c) Bei der Phosphataseaktivität handelt es sich um die hydrolytische Entfernung der Phosphatgruppe des phosphorylierten Antwortregulators durch die Histidinkinase. (Stock et al. 2000)

Das vom Sensor wahrgenommene Signal wird über *coiled-coil* Verbindungshelices an den HK-Effektor weitergegeben. Die *coiled-coil* α -Helices winden sich dabei um eine zentrale Achse und besitzen sich wiederholende Aminosäureabfolgen, die die Ausbildung dieses Strukturmotives garantieren. Die Aminosäuren der einen Helix greifen dabei optimal in die Lücken der anderen Helix und umgekehrt. Kommt es zu einer Signaländerung im Sensor so wird diese im Helixbündel vermutlich durch Rotation übertragen. Die Packung der Aminosäuren ändert sich und die daraus resultierenden Verzerrungen der Helices werden an die α_1 und α_2 Helices der DHp Domäne übertragen (Möglich 2019).

Abhängig von der Orientierung der Helices befindet sich das für die Aktivität verantwortliche Histidin entweder im Kinasezustand an der Oberfläche oder im Phosphatasezustand im Inneren des Helixbündels (Abbildung 11). Der Kinasezustand erlaubt es, dass das Histidin entweder in der Nähe der CA Domäne des eigenen Monomers oder des benachbarten Monomers ist. Als Resultat erfolgt Autophosphorylierung entweder in *cis* oder in *trans* Orientierung (Ashenberg et al. 2013). Obwohl die CA Domäne sehr variable Anordnungen einnehmen kann, ist sie während der Autophosphorylierungsreaktion in einer spezifischen Orientierung zur DHp Domäne, die eine räumliche Nähe der γ -Phosphatgruppe des ATP-Kofaktors zum katalytisch aktiven Histidin gestattet (Bhate et al. 2015). Zur Übertragung der Phosphatgruppe bilden das aktive Histidin und ein benachbarter Asparaginsäurerest eine katalytische Diade in DesK. Die CA Domäne löst sich vermutlich nach der Autophosphorylierung von der DHp Domäne und schafft Platz für die Interaktion mit dem Antwortregulator (Trajtenberg et al. 2016). Im Kinasezustand erfolgt demnach sowohl die Autophosphorylierung als auch die Übertragung der Phosphatgruppe auf den Antwortregulator (Trajtenberg et al. 2016; Buschiazzo und Trajtenberg 2019). Der Transfer der Phosphatgruppen erfolgt dabei sehr spezifisch nur auf Antwortregulatoren und nicht auf andere Proteine (Capra et al. 2010), jedoch konnte gezeigt werden, dass Histidinkinasen auch Antwortregulatoren aus anderen Organismen phosphorylieren können (Bhoo et al. 2001).

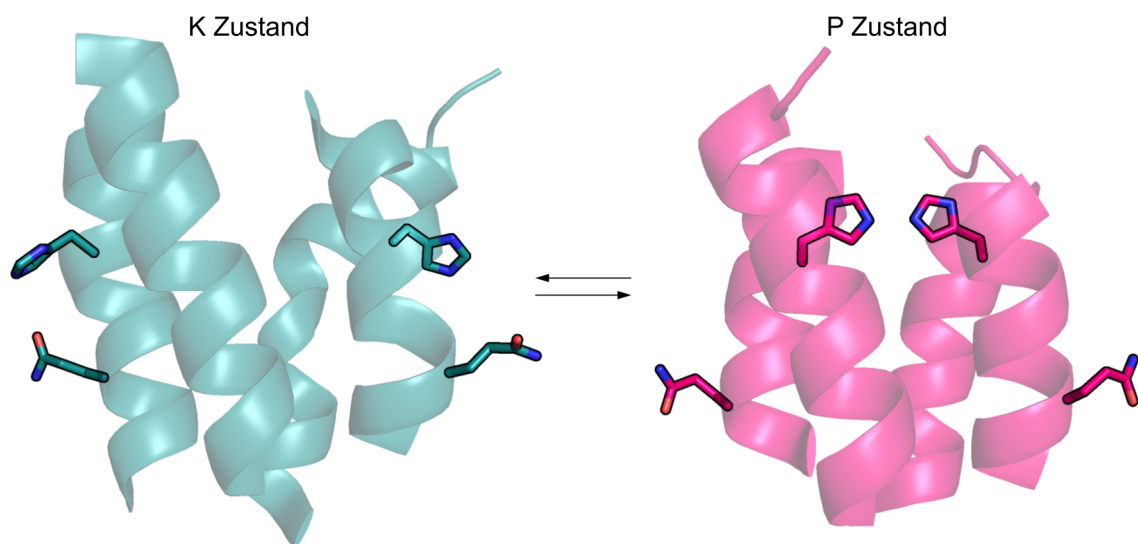


Abbildung 11: Modell für die Positionierung der katalytisch relevanten Aminosäuren in der DHp Domäne. Die katalytisch aktiven Histidine sind im Kinasezustand (K Zustand) an der Oberfläche des Helixbündels. Nach Übergang in den Phosphatasezustand (P Zustand) befinden sich die Histidine im inneren Bereich zwischen den Helices und das Glutamin kann an der Phosphatasereaktion mitwirken. (erstellt aus PDB-Einträgen 5ium und 5iun)

Im Phosphatasezustand kommt es zur Rotation der α -Helices der DHP Domäne. Das aktive Histidin verlagert sich nun ins Zentrum des Helixbündels und ein Glutamin tritt an dessen Position an der Oberfläche (Abbildung 11). Die drei wichtigsten Aminosäuren für die Histidinkinasefunktionen bilden ein hochkonserviertes H-D-x-x-x-Q Motiv in der Pfam HisKA_3 Klasse (Finn et al. 2006; Willett und Kirby 2012), jedoch gibt es auch Abweichungen von diesem Motiv (Diensthuber et al. 2013). Unabhängig von Signalzustand bleibt die Bindestelle für den Antwortregulator größtenteils unverändert. Nachdem der Antwortregulator an die Histidinkinase im Phosphatasezustand bindet, erfolgt die Hydrolyse durch ein vom Glutamin koordiniertes Wassermolekül und der Antwortregulator wird anschließend freigegeben.

1.1.4 Lichtgesteuerte Protein-Protein Interaktion in Pflanzen

Die biologische Funktion pflanzlicher Phytochrome ist deutlich ausgiebiger untersucht als die Rolle der entsprechenden Phytochrome in anderen Organismen. Zu den wichtigsten Prozessen, die durch pflanzliche Phytochrome beeinflusst werden, zählen Samenkeimung (Song und Choi 2019), Wurzelwachstum (Gil et al. 2018; Ha et al. 2018), Photomorphogenese (Tripathi et al. 2019; Enderle et al. 2017), rotlichtabhängige Seneszenz von Blättern (Lim et al. 2018), die Wahrnehmung der Morgendämmerung (Seaton et al. 2018; Qiu et al. 2017), Beeinflussung des zirkadianen Rhythmus (Oakenfull et al. 2019; Oakenfull und Davis 2017; Shor et al. 2017), Schattenvermeidung und Detektion von Nachbarpflanzen (Romero-Montepaone et al. 2020; Roig-Villanova et al. 2019). Des Weiteren konnte gezeigt werden, dass das Pflanzenwachstum, die Biomassenproduktion und der Ernteertrag von Mais durch Phytochrom B kontrolliert wird (Wies et al. 2019). Pflanzliche Phytochrome fungieren dabei sowohl als Sensoren für Licht als auch für Temperatur (Han et al. 2019; Klose et al. 2020).

Auf molekularer Ebene werden diese Prozesse in der Regel über die Veränderung der Expression verschiedenster Gene gesteuert (Ushijima et al. 2017; Pham et al. 2018; Stawska und Oracz 2019). Der durch Phytochrome gesteuerte Kontrollmechanismus in Pflanzen beinhaltet dabei ein ausgeklügeltes Zusammenspiel von Proteinsynthese, photochemischer Signalwahrnehmung, Translokation in den Zellkern, Ausbildung von *photobodies*, thermische Reversion und Proteinabbau (Sellaro et al. 2019; Enderle et al. 2017; Grima et al. 2018; Hahm et al. 2020; Helizon et al. 2018). Phytochrome befinden sich im Dunkeln zunächst im Zytosol, werden jedoch nach Rotlichtabsorption in den Zellkern transportiert (Abbildung 12). Die biologische Funktion wird anschließend über lichtabhängige Protein-Protein Interaktion (PPI) mit Partnerproteinen, primär Phytochrominteraktionsfaktoren (*phytochrome interacting*

factors, PIFs), vermittelt (Pham et al. 2018). PIFs sind *basic Helix-loop-helix* (bHLH) Transkriptionsfaktoren, deren lichtgesteuerte Interaktion mit Phytochromen zum Übergang von Skotomorphogenese zu Photomorphogenese führt und zur Folge hat, dass PIF Proteine durch Phosphorylierung für den Proteinabbau freigegeben werden (Castillon et al. 2007; Leivar und Quail 2011).

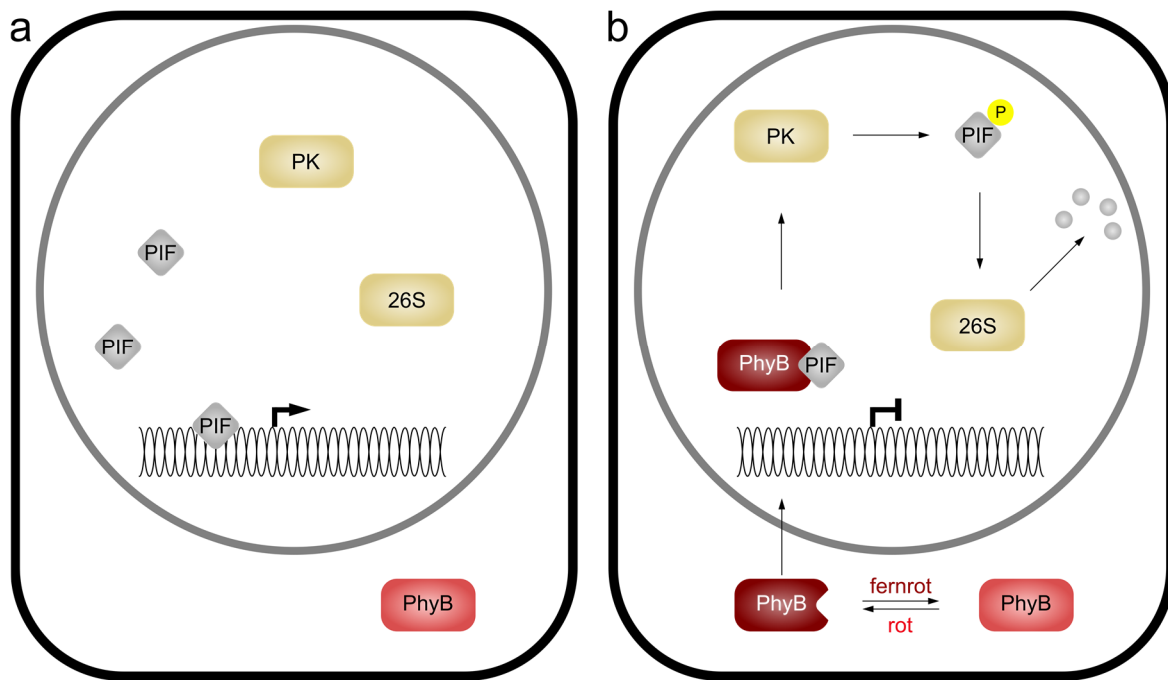


Abbildung 12: Dynamische Regulation der Konzentration der Phytochrominteraktionsfaktoren (PIF) in pflanzlichen Zellen. (a) Während der Skotomorphogenese liegt Phytochrom B (PhyB) im Pr Zustand vor und befindet sich im Zytosol. PIF Proteine im Zellkern können an die DNS binden und die Expression von Genen, die wichtig sind für das Wachstum in Dunkelheit sind, initiieren. (b) Die Absorption von Rotlicht bewirkt den Übergang von Skotomorphogenese zu Photomorphogenese. PhyB wird in den Pfr Zustand überführt und anschließend in den Zellkern transportiert. Dort angekommen kommt es zur Wechselwirkung mit PIF Proteinen und die Genexpression wird gestoppt. Die PhyB-PIF Interaktion bewirkt, dass PIF Proteine von PhyB und Proteinkinasen (PK) phosphoryliert werden. Die phosphorylierten PIFs werden nach anschließender Ubiquitinierung im 26S-Proteasom (26S) abgebaut (Castillon et al. 2007; Pham et al. 2018).

Pflanzliche Phytochrome besitzen anstelle der Histidinkinase Domäne eine Histidinkinase-ähnliche Domäne (*histidine kinase related domain, HKRD*) bei der das konservierte Histidin im aktiven Zentrum fehlt. Anstelle der Histidinkinaseaktivität ist eine Serin/Threonin-Kinaseaktivität berichtet worden (Yeh und Lagarias 1998) und die Phosphorylierung einiger Zielproteine durch pflanzliche Phytochrome wurde nachgewiesen (Nagano 2016). Interessanterweise konnte für *Avena sativa* Phytochrom A festgestellt werden, dass lediglich das Photosensormodul ausreichend ist um PIF3 zu phosphorylieren (Shin et al. 2016). Die Stärke des Phosphorylierungseffekts von Phytochromen ist jedoch in vielen Fällen unklar und

es wird vermutet, dass die Hyperphosphorylierung, die bei PIF Proteinen in Pflanzen nachweislich zum Proteinabbau führt, auch auf andere Proteinkinasen zurückzuführen ist (Hoang et al. 2019; Ni et al. 2017; Pham et al. 2018).

Die lichtkontrollierte Interaktion von pflanzlichen Phytochromen und PIF Proteinen, von der erstmals vor über 20 Jahren berichtet wurde (Ni et al. 1998; Ni et al. 1999), ist die am besten untersuchte biologische Funktion von Phytochromen. Phytochrome und PIF Proteine wurden mittlerweile in verschiedensten Pflanzenorganismen nachgewiesen (Pham et al. 2018; Lee und Choi 2017). Als Model für diese Interaktionsfamilie wird das *Arabidopsis thaliana* Phytochrom B (*AtPhyB*) und PIF System herangezogen, für das bereits tiefgreifendere biochemische und biologische Charakterisierungen durchgeführt wurden. *A. thaliana* verfügt über insgesamt 8 PIF Proteine (PIF1-8), die alle mit *AtPhyB* interagieren (Pham et al. 2018; Lee und Choi 2017). Zusätzlich dazu sind PIF1 und PIF3 in der Lage mit *A. thaliana* Phytochrom A (*AtPhyA*) zu interagieren (Huq et al. 2004; Lee und Choi 2017), wobei die Interaktion präferenziell mit dem Pfr Zustand von Phytochromen erfolgt (Ni et al. 1999; Huq et al. 2004; Khanna et al. 2004; Huq und Quail 2002; Leivar und Monte 2014). Im dunkeladaptierten Pr Zustand findet hingegen keine bzw. nur geringe Interaktion statt (Abbildung 13a).



Abbildung 13: Schematische Darstellung der lichtkontrollierten *AtPhyB*-PIF Interaktion. (a) Nach Rotlichtbestrahlung interagieren *AtPhyB* und PIF. Die Wechselwirkung kann durch Fernrotlicht aufgehoben werden. (b) PIFs sind *basic Helix Loop Helix* (bHLH) Transkriptionsfaktoren, die ein N-terminales „aktives Phytochrom B“ Bindemotiv (APB) besitzen. Zusätzlich besitzen PIF1 und PIF3 noch ein „aktives Phytochrom A“ Bindemotiv (APA).

Für die spezifische Interaktion mit *AtPhyA* bzw. *AtPhyB* sind die aktiven Phytochrombindemotive A bzw. B (*active phytochrome A/B binding*, APA bzw. APB) in PIF Proteinen verantwortlich (Leivar und Quail 2011; Khanna et al. 2004), die in den N-terminalen Bereichen der PIF Proteine konserviert sind (Abbildung 13b). Besonders das APB Motiv der PIFs wurde bereits näher charakterisiert. So konnte gezeigt werden, dass das Motiv in den ersten 100 Aminosäuren aller PIFs vorliegt und dort in zwei unterschiedlich stark konservierte Bereiche eingeordnet werden kann (Khanna et al. 2004). Der erste, N-terminal liegende Bereich, beinhaltet das hochkonservierte E-L-x-x-x-G-Q Motiv, von dem in Mutationsstudien gezeigt wurde, dass es unentbehrlich für die Interaktion mit *AtPhyB* ist

(Khanna et al. 2004). Über den zweiten, weniger stark konservierten Bereich und dessen Aufgabe bei der Interaktion mit PhyB ist aktuell jedoch noch wenig bekannt. Des Weiteren ist auffällig, dass es deutliche Unterschiede in den nicht konservierten Bereichen der ersten 100 Aminosäuren gibt. Beim Großteil der PIF Proteine befinden sich noch einige N-terminale Aminosäuren vor dem ersten konservierten Bereich, jedoch variiert ihre Anzahl stark zwischen den einzelnen Vertretern der Familie. Außerdem weist das Verbindungsstück zwischen dem vorderen und dem hinteren konservierten Bereich kaum Übereinstimmung zwischen unterschiedlichen PIFs auf, und auch dessen Länge unterscheidet sich deutlich innerhalb der Proteinfamilie (Khanna et al. 2004).

Als die *AtPhyB*-PIF Interaktion erstmals entdeckt wurde, vermutete man, dass PIF Proteine mit dem C-terminalen Effektorteil von *AtPhyB* interagieren (Ni et al. 1998). Dies wurde anhand von Mutationsstudien innerhalb des Effektors geschlussfolgert (Quail et al. 1995; Wagner und Quail 1995; Xu et al. 1995), jedoch zeigte sich bald, dass der N-terminale Sensorteil primär für die Interaktion mit PIFs verantwortlich ist (Ni et al. 1999; Shimizu-Sato et al. 2002; Matsushita et al. 2003). Der Grund für diese fälschliche Annahme ist vermutlich darin begründet, dass Mutationen innerhalb der beiden PAS Domänen des Effektors zum Verlust der Fähigkeit von Phytochromen führt in den Zellkern transportiert zu werden (Bae und Choi 2008). Infolgedessen kann die Interaktion mit PIF Proteinen aufgrund räumlicher Trennung nicht stattfinden, obwohl diese auf molekulare Ebene in Theorie noch möglich sein sollte (Nagano 2016). Für eine Funktionalität der Interaktion mit PIFs ist es notwendig, dass das Sensormodul von *AtPhyB* vollständig vorliegt, da die Entfernung der PHY Domäne den Pfr Zustand destabilisiert (Hill et al. 1994). Die genaue Interaktionsstelle von PIF Proteinen am *AtPhyB* ist aktuell noch unbekannt, jedoch deuten Mutationsstudien darauf hin, dass die N-terminale Knotenregion zwischen PAS und GAF Domäne einen entscheidenden Einfluss auf die Interaktion hat (Oka et al. 2008; Kikis et al. 2009). Interessanterweise verlangsamt die Bindung von *AtPhyB*-PIF Proteinen die thermische Reversion des Pfr Zustands, was wiederum die Rolle des *AtPhyB* als Licht und Temperatursensor unterstreicht. (Smith et al. 2017; Klose et al. 2020). Neben der *AtPhyB*-PIF Interaktion sind vor allem lichtabhängige Interaktionen von *AtPhyA* mit Partnerproteinen bekannt (Lu et al. 2015; Sheerin et al. 2015), jedoch interagieren diese Partner oftmals auch mit *AtPhyB* ähnlich wie es beispielsweise bei der Interaktion mit PIF1 bzw. PIF3 der Fall ist (Zhu et al. 2000; Pham et al. 2018).

Für lange Zeit wurde angenommen, dass die lichtgesteuerte Interaktion von Phytochromen und Partnerproteinen eine spezifische Eigenschaft pflanzlicher Phytochrome ist. Jedoch konnte

gezeigt werden, dass das Bakteriophytochrom RpBphP1 aus *Rhodopseudomonas palustris* lichtabhängige Interaktion mit dem Transkriptionsrepressor RpPpsR2 eingeht (Kojadinovic et al. 2008; Bellini und Papiz 2012).

Lichtgesteuerte Systeme besitzen nicht nur eine hohe biologische Relevanz, sondern haben auch einen hohen Wert für die Anwendung in der synthetischen Biologie und Biotechnologie, welcher im Folgenden näher erörtert werden soll.

1.2 Optogenetik und Biotechnologie

Die Kontrolle biochemischer, biophysikalischer und zellulärer Prozess mit Hilfe von Licht bietet oftmals entscheidende Vorteile gegenüber klassischen konstitutiv aktiven bzw. chemisch induzierten Verfahren. Sie erlaubt eine hohe räumliche als auch zeitliche Auflösung, ist nicht invasiv, reversibel und wiederholt einsetzbar. Die genetische Veränderung von Zellen für die Expression von Photorezeptorproteinen und der damit verbundenen lichtgesteuerten Manipulation des Verhaltens und der Physiologie von Zellen wird als Optogenetik bezeichnet (Deisseroth et al. 2006; Deisseroth 2011). Basierend auf natürlichen Photorezeptoren wurde über die Jahre ein Repertoire an künstlichen Photorezeptoren erstellt (Möglich und Moffat 2010; Ziegler und Möglich 2015; Losi et al. 2018; Chernov et al. 2017). Der Ursprung dieser Technologie ist in der Entdeckung von lichtkontrollierten Kanalrhodopsinen in einzelligen Algen begründet (Nagel et al. 2002; Nagel et al. 2003). Daraus resultierend ergab sich, dass diese Technologie zunächst primär Einsatz in der Neurobiologie fand, um beispielsweise Depolarisation in Nervenzellen zu induzieren (Boyden et al. 2005; Gradinaru et al. 2008). Dieses, als neuronale Optogenetik bekannte Forschungsgebiet, stellt mittlerweile nur einen Teil der Anwendungsmöglichkeiten optogenetischer Systeme dar. Inzwischen wird diese Technologie in vielen zellulären Kontexten zur Kontrolle und Erforschung unzähliger Signalwege angewandt (Losi et al. 2018).

Während die Anwendung von Phytochromen lange Zeit vor allem in der Optogenetik beheimatet war, findet diese Photorezeptorfamilie indes auch vermehrt Einsatz in der Biotechnologie zur Kontrolle von Prozessen und Materialien (Hörner et al. 2019a; Hörner et al. 2019b; Hudson 2018; Jia et al. 2018; Beyer et al. 2018). Auch die Erzeugung von Fluoreszenzproteinen, Biosensoren und biologischen Reportern, die auf Grundlage der Phytochrome entwickelt wurden, ist rapide vorangetrieben worden. Im Folgenden soll daher

speziell auf die Anwendungsmöglichkeit phytochrombasierter Systeme für Biotechnologie und Optogenetik eingegangen werden.

1.2.1 Fluoreszenzproteine und Biosensoren

Ausgehend von der Entdeckung des grün fluoreszierenden Proteins (*green fluorescent protein*, GFP) im Jahre 1962 (Shimomura et al. 1962) ist die Weiterentwicklung und Anwendung von Fluoreszenzproteinen rasant vorangeschritten. Fluoreszenzproteine sind nunmehr als biotechnologisches Werkzeug für die Forschung unentbehrlich (Rodriguez et al. 2017). Trotz der Tatsache, dass natürliche und künstliche Fluoreszenzproteine basierend auf GFP und homologen Proteinen annähernd das gesamte Spektrum des Lichts abdecken, konnten erst durch die Modifikation von bakteriellen Phytochromen (Nah-)Infrarotfluoreszenzproteine entwickelt werden. Besonders für die Anwendung in tierischem Gewebe besitzen Infrarotfluoreszenzproteine entscheidende Vorteile, so ist die Eindringtiefe des benötigten Anregungslichts höher und der Streulichtanteil minimiert (Jöbsis 1977; Weissleder und Ntziachristos 2003). Das erste auf Phytochromen basierende Fluoreszenzprotein wurde aus dem cyanobakteriellen Cph1 entwickelt und verwendete den cyanobakterienspezifischen Chromophor PCB (Fischer und Lagarias 2004). Erst durch Erzeugung der Fluoreszenzproteine IFP1.4 und iRFP aus den Bakteriophytochromen *D. radiodurans* bzw. *R. palustris* fand diese Familie der Fluoreszenzproteine Anwendung in Säugetiergewebe, da als Chromophor das in Säugern vorhandene BV verwendet werden konnte (Shu et al. 2009; Filonov et al. 2011; Tran et al. 2014). Cyanobakterielle Fluoreszenzproteine wurden indes ebenfalls weiterentwickelt, um BV als Chromophor zu verwenden was ihr Anwendungspotenzial signifikant steigert (Rodriguez et al. 2016). Während BV zwar universell in Säugerzellen vertreten zu sein scheint (Tran et al. 2014; Gasser et al. 2014), sei jedoch angemerkt, dass abhängig vom verwendeten Fluoreszenzprotein und vom Zielgewebe ggf. die Zugabe von exogenem BV notwendig ist (Chernov et al. 2017). Um die Fluoreszenzintensität von Phytochromen zu steigern muss der lichtgetriebene Pr → Pfr Übergang inhibiert werden. (Chernov et al. 2017). Dies wird entweder durch das Entfernen von Domänen (Shu et al. 2009) oder durch das Einbringen von Mutationen (Fischer et al. 2005; Su und Lagarias 2007), z.B. Y276H in *A. thaliana* PhyB (Abbildung 5), erreicht. Diese Tyrosin zu Histidin Substitution in *AtPhyB* hält den Chromophor spektral im Pr Zustand gefangen, wobei das Protein sich funktional wie im Pfr Zustand verhält (Su und Lagarias 2007).

Phytochrombasierte Reporter und Biosensoren fußen nach aktuellem Stand auf Fluoreszenzproteine aus Bakteriophytochromen. Es gibt dabei zwei Varianten um diese als Reporter zu verwenden. In einem aktuell noch weniger häufig verwendeten Ansatz wird der Förster-Resonanzenergietransfer (FRET) zwischen zwei (Nah-)Infrarotfluoreszenzproteinen ausgenutzt. Eine mögliche Art der Anwendung ist dabei die Untersuchung von Proteinwechselwirkungen wie beispielsweise die Interaktion von Rac1 und der p21-binde Domäne von PAK1 (Shcherbakova et al. 2018).

Die zweite, deutlich häufiger verwendete, Variante bei diesen Reportern beruht auf der Spaltung eines (Nah-)Infrarotfluoreszenzproteins zwischen PAS und GAF Domäne. Die Trennung der beiden Domänen voneinander bewirkt einen Verlust der Fluoreszenz. Wenn die beiden Fragmente aber beispielsweise an zwei unterschiedliche, interagierende Proteine gekoppelt werden, kann die Fluoreszenz durch die Erzeugung von räumlicher Nähe wieder hergestellt werden (Filonov et al. 2012; Tchekanda et al. 2014; Chen et al. 2015; Pandey et al. 2015). Eine weitere mögliche Anwendung dieser Methodik wurde beim iProtease Reporter verwendet (To et al. 2015). Das iProtease System beinhaltet die PAS und die GAF Domäne des Fluoreszenzreporters, jedoch sind diese über eine Proteaseschnittstelle so miteinander verbunden, dass kein kovalenter Einbau des Chromophoren stattfinden kann. Erst nach Spaltung der Schnittstelle durch die entsprechende Protease wird das funktionale Fluoreszenzprotein gebildet.

1.2.2 Lichtaktivierbare Enzyme

Eine Vielzahl von rotlichtaktivierbaren Enzymen sind nach dem Prinzip des *photoreceptor engineering* erzeugt worden (Ziegler und Möglich 2015). Gezielter Austausch von natürlichen Effektordomänen durch Proteindomänen mit alternativer Funktion ermöglicht es lichtaktivierbare Enzyme zu erzeugen. Es ist dabei jedoch zu beachten, dass es strukturellen Übereinstimmungen zwischen natürlichem und gewünschtem Effektor gibt. Trotz dieser Einschränkung war es möglich in den letzten Jahren einige rotlichtabhängige Enzyme auf Grundlage von Bakteriophytochromen zu entwickeln.

Der Schwerpunkt bei der Erzeugung rotlichtaktivierbarer Enzyme lag größtenteils auf der Bildung und dem Abbau von zyklischen Nukleotiden, die als *second messenger* physiologische Prozesse in Prokaryoten und Eukaryoten beeinflussen (Steiner et al. 2002). Photoaktivierbare Adenylylcyclasen (PACs) wurden zunächst in *Euglena gracilis* entdeckt und reagieren auf Blaulicht (Iseki et al. 2002). Mit der Intention PACs rotlichtkontrollierbar zu machen wurde

Ilac aus dem Photosensor des *Rhodobacter sphaeroides* Bakteriophytochroms und der Adenylylzyklasedomäne CyaB1 aus *Nostoc sp.* geschaffen (Ryu et al. 2014). Seither wurde die gezielte Entwicklung und Erzeugung rotlichtaktivierbarer Zyklasten weiter vorangetrieben und umfasst nun neben spezifischen Adenylyl- und Guanylylzyklasen auch Diguanylylzyklasen (Gourinchas et al. 2017; Etzl et al. 2018). Kürzlich wurden PACs durch die Anwendung programmierbare lichtemittierender Diodenmatrixsysteme näher charakterisiert und deren Lichtantwort optimiert (Stüven et al. 2019; Dietler et al. 2019). Eine lichtaktivierbare Phosphodiesterase (LAPD) stellt das Gegenstück zu den PACs dar. Durch Fusion des Sensormoduls von *D. radiodurans* und der menschlichen Phosphodiesterase PDE2A katalysiert LAPD die Hydrolyse von cAMP und cGMP (Gasser et al. 2014). Kürzlich wurde eine Vielzahl analoger lichtaktivierbarer PDEs vorgestellt, die zum Teil verbesserte Lichtantwort und Reversibilität zeigen (Stabel et al. 2019). Des Weiteren wurde gezeigt, dass es möglich ist zyklische Nukleotidkonzentration in Zellen gezielt und präzise unter Zuhilfenahme von LAPD und bPAC zu kontrollieren (Raju et al. 2019).

Obwohl Bakteriophytochrome als Photorezeptorhistidinkinasen fungieren, ist ihre Anwendung als Kinasen in der Optogenetik eher selten. So wurde beispielsweise ein Grün- bzw. Rotlicht steuerbarer Transkriptionsregulationssignalweg basierend auf der Kinaseaktivität von CBCRs genutzt, um Genexpression in *Bacillus subtilis* lichtabhängig zu steuern (Castillo-Hair et al. 2019). In einem anderen Ansatz wurde das Photosensormodul von *D. radiodurans* mit Tyrosinkinasen kombiniert, um Signalwege in Zellen rotlichtabhängig zu manipulieren (Leopold et al. 2020).

1.2.3 Lichtkontrollierbare Protein-Protein Interaktion

Die Kontrolle von Protein-Protein Interaktionen (PPI) mit Hilfe von Rot- und Fernrotlicht ist eines der ältesten bekannten Anwendungen für Phytochrome in der synthetischen Biologie (Shimizu-Sato et al. 2002). Lichtabhängige PPIs können in Homo- und Heterodimerisierung unterteilt werden (Tichy et al. 2019). Während rotlichtgesteuerte Homodimerisierung zur Kontrolle von Signalwegen erst kürzlich Einzug in das optogenetische Repertoire fanden (Reichhart et al. 2016), ist vor allem die Anwendung des *AtPhyB*-PIF Systems als Heterodimerisierungswerkzeug weit verbreitet (Abbildung 14). In der Regel werden verkürzte Fragmente der beiden Interaktionspartner eingesetzt. So genügt es für ein funktionelles System die NTE und das photosensorische Modul von *AtPhyB* zusammen mit den ersten 100 Aminosäuren der PIF Konstrukte zu verwenden (Levskaya et al. 2009). Besonders die präzise

und reversible Steuerung der *AtPhyB*-PIF Interaktion mittels Lichts unterschiedlicher Wellenlängen stellt einen entscheidenden Vorteil gegenüber anderen Photorezeptorsystemen dar, bei denen die PPI nur über längere Verweilzeit in Dunkelheit aufgehoben wird (Adrian et al. 2017).

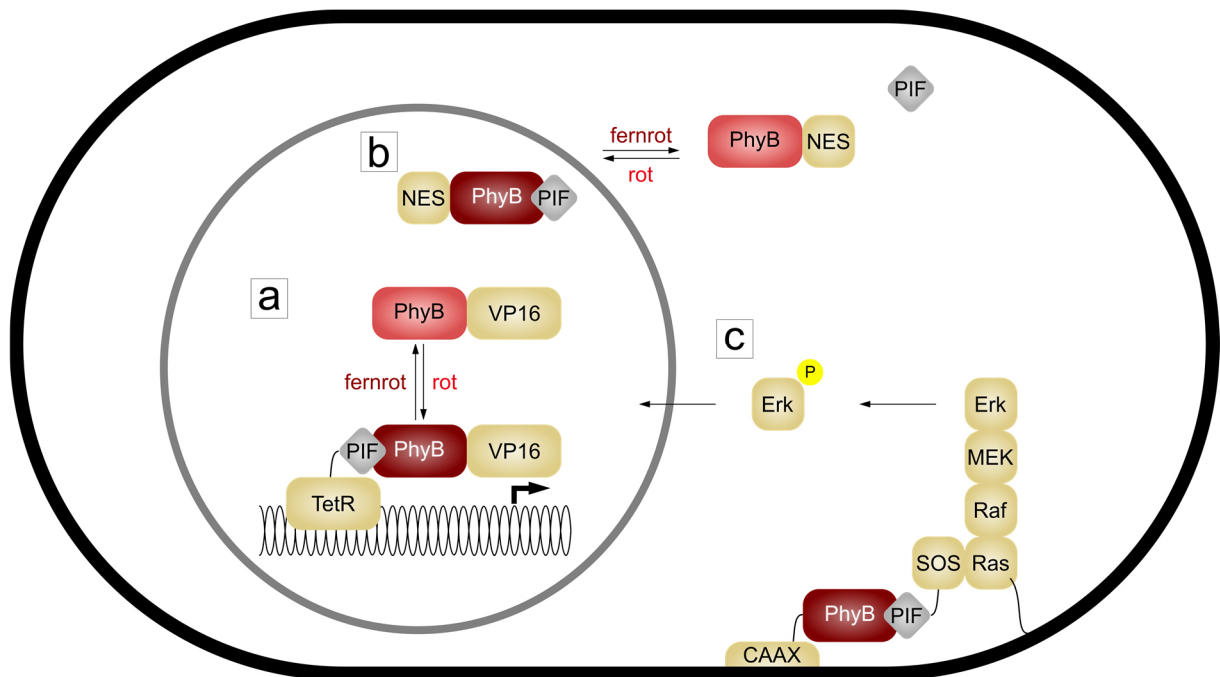


Abbildung 14: Übersicht der wichtigsten Einsatzmöglichkeiten des lichtschaltbaren *AtPhyB*-PIF Interaktionssystems in Zellen. (a) Lichtkontrollierte Genexpression. *AtPhyB* ist gekoppelt an eine VP16 Transaktivator-domäne und PIF an den Tet Repressor (TetR). Rotlicht bewirkt Transkription des Zielgens während Fernrotlicht diese inhibiert. (b) Subzelluläre Lokalisierung. *AtPhyB* fusioniert mit einem nuklearen Exportsignal (NES) wird durch rotlichtinduzierte *AtPhyB*-PIF Interaktion in den Zellkern befördert. Fernrotlicht unterbricht die Interaktion und es kommt zum Export aus dem Nukleus. (c) Lichtgesteuerte Signalwege. *AtPhyB* ist mittels CAAX Motiv an der Plasmamembran gebunden. Im interagierenden Zustand kommt es zur Lokalisierung von PIF-SOS an *AtPhyB* was die Aktivierung des Ras/Erk Signalwegs zur Folge hat. Fernrotlicht hebt die Interaktion auf und stoppt die Aktivierung des Signalweges.

Ein prominentes Beispiel für die Nutzung des *AtPhyB*-PIF Systems ist die lichtabhängige Regulierung von Genexpression (Müller et al. 2013a; Müller et al. 2014). In diesem System ist *AtPhyB* an eine Transaktivator-domäne gekoppelt und PIF, z.B. PIF6, wurde mit einem DNA-bindenden Protein, z.B. dem Tet Repressor Protein (TetR) verbunden (Abbildung 14a). Das TetR-PIF Konstrukt bindet kontinuierlich an den TetA Operator auf einem Reporter-DNS-Konstrukt. Die Position des TetA Operators ermöglicht dabei räumlicher Nähe des TetR-PIFs zu einer minimal Promotorsequenz, welche die Expression des Zielgens kontrolliert. Erst durch Bestrahlung mit Rotlicht wird *AtPhyB*-VP16 in seinen interagierenden Zustand überführt,

bindet an den Repressor und führt zur Transkription des Zielgens. Die Genexpression kann durch Bestrahlung mit Fernrotlicht unterbunden werden. Durch die Kombination von Photorezeptorsystemen mit unterschiedlicher Wellenlängenspezifität ist es unter anderem möglich die Expression verschiedenster Gene parallel und unabhängig voneinander zu kontrollieren (Müller et al. 2013b).

Ein weiteres Anwendungsgebiet ist subzelluläre Lokalisierung (Abbildung 14b und 14c). Ein oftmals verwendetes Verfahren ist die Fusion von *AtPhyB* und der CAAX Transmembranlokalisierungssequenz (Levskaya et al. 2009; Toettcher et al. 2011a). Alternativ können auch andere Ankerproteine verwendet werden, um *AtPhyB* an die verschiedensten subzellulären Positionen zu befördern (Yang et al. 2013). Interessanterweise kann für eine Lokalisierung in den Zellkern auch die entsprechende intrinsische Funktion von *AtPhyB* und PIF genutzt werden (Abbildung 14b). Kombiniert man *AtPhyB* zusätzlich mit einem nuklearen Exportsignal kann ein lichtkontrollierbares Transportsystem zwischen Cytoplasma und Zellkern geschaffen werden (Beyer et al. 2015). Durch die Fusion von PIFs mit Zielproteinen wird es ermöglicht die Lokalisierung dieser Proteine in Abhängigkeit der Lichtbestrahlung zu regulieren (Buckley et al. 2016). Eine derartige Anwendung erlaubt es außerdem Signalwege in Zellen lichtgesteuert zu manipulieren (Abbildung 14c). Durch Verbindung von PIF und Guaninnukleotid-Austauschfaktoren ist beispielweise die präzise räumliche Aktivierung von GTPasen möglich (Levskaya et al. 2009). Des Weiteren konnte durch die Kombination von membrangebundenem *AtPhyB*-CAAX und PIF6-SOS ein lichtgesteuertes Werkzeug für die Erforschung des Ras/Erk Signalwegs erzeugt werden (Toettcher et al. 2013; Goglia et al. 2017). Nach Bestrahlung mit Rotlicht wird SOS an die Plasmamembran transportiert und aktiviert dort Ras. Dies führt wiederum zur Phosphorylierung von Erk, das anschließend in den Zellkern transportiert wird. Dieser Prozess kann durch Bestrahlung mit Fernrotlicht rückgängig gemacht werden (Toettcher et al. 2013; Goglia et al. 2017).

Das *AtPhyB*-PIF System benötigt für die Ausübung seiner Funktion den P Φ B oder PCB Chromophor. Da tierische Zellen und Modelorganismen diesen Chromophoren in der Regel nicht intrinsisch produzieren, ist es notwendig das jeweilige System extern über das Medium mit PCB zu versorgen (Müller et al. 2013a) oder durch genetische Manipulation die Enzyme für die PCB Synthese einzubringen (Hochrein et al. 2017). Durch die Entdeckung der lichtkontrollierbaren bakteriellen PPI aus *R. palustris* gibt es mittlerweile ein Alternative zum *AtPhyB*-PIF System, das mit zelleigenem BV verwendet werden kann (Kaberniuk et al. 2016). Aufgrund der spektralen Eigenschaften bakterieller Phytochrome ist die Lichtempfindlichkeit

des Systems weiter in den Fernrot- bis Nahinfrarotlichtbereich verschoben. Außerdem wurde das Anwendungspotenzial des Systems durch Verringerung der Proteingrößen optimiert (Redchuk et al. 2017). Mittlerweile wurde gezeigt, dass es möglich ist dieses bakterielle System analog zum *AtPhyB*-PIF Interaktionspaar für lichtkontrollierte Steuerung verschiedenster Prozesse in Zellen zu verwenden (Redchuk et al. 2018a; Redchuk et al. 2018b; Redchuk et al. 2020).

Neben der Anwendung in der Optogenetik findet das *AtPhyB*-PIF System indes vermehrt Einsatz innerhalb der Biotechnologie. Durch die Kopplung von *AtPhyB* an Membranen und die präzise Bestrahlung mit Rotlicht ist es beispielsweise möglich Membranoberflächen mit PIF Fusionsproteinen zu beschichten (Jia et al. 2018). Die lokale Konzentration dieser PIF-Schicht kann dabei verhältnismäßig einfach durch Veränderung der Laserleistung und der Bestrahlungszeit angepasst werden (Jia et al. 2018). Die *AtPhyB*-PIF Interaktion kann alternativ auch genutzt werden, um Biomoleküle innerhalb von Systemen freizusetzen oder diese aus den entsprechenden Anwendungsbereichen zu entfernen (Beyer et al. 2018). Perspektivisch können verkürzte PIF-tags für Proteinreinigungen eingesetzt werden um Zielproteine aus Lysatlösungen zu extrahieren (Hörner et al. 2019a). Zusätzlich ist die reversible Veränderung der mechanischen Eigenschaften von Biomaterialien denkbar, indem deren Durchlässigkeit und Festigkeit sich beispielweise durch *AtPhyB*-PIF Interaktion manipulieren lässt (Hörner et al. 2019b). Ein für Forschung und Biotechnologie hoch relevantes Einsatzgebiet ist auch die Veränderung von Zelloberflächen. Zellen, die an ihrer Oberfläche *AtPhyB* präsentieren, können genutzt werden um PIF beschichtete Oberflächen in reversibler Weise zu besetzen (Yüz et al. 2018; Baaske et al. 2019). Von hohem Interesse ist dieser Oberflächenveränderungsansatz für die Untersuchung der Wirkungsweisen von T-Zellen (Yousefi et al. 2019).

1.3 Zielsetzung

Phytochrome sind sowohl für die Lichtantwort von Organismen als auch die Anwendung innerhalb der Optogenetik und Biotechnologie von hohem Interesse. Die Charakteristika und Wirkweisen der Phytochrome aus verschiedenen Organismenfamilien sind vielfältig. Ziel dieser Arbeit war es daher das Verständnis der verschiedenen Eigenschaften und Wechselwirkungen von Phytochromen zu erweitern, und mit diesem Wissen neue Anwendungsmöglichkeiten für phytochrombasierte Systeme zu eröffnen.

Besonders das Phytochrom B aus *A. thaliana* fand in den letzten Jahren vielfältigen Einsatz in der Optogenetik. Obwohl die lichtgesteuerte Interaktion dieses Phytochroms mit seinen PIF Partnerproteinen vielseitig ausgenutzt wurde, um zelluläre Prozesse zu manipulieren, ist überraschend wenig über die eigentliche Interaktion dieser Proteine bekannt. Um diese Lücke zu schließen, soll die Interaktion von *AtPhyB* und PIFs näher untersucht werden. Es werden daher Interaktionsstärken von verschiedenen natürlichen und modifizierten PIF Varianten mit den lichtabhängigen Zuständen von *AtPhyB* bestimmt, um eine detaillierte Charakterisierung des für die Interaktion notwendigen APB Motivs von PIF Proteinen zu erhalten. Jedoch ist neben der Interaktionsstärke auch die Dynamik der Wechselwirkung zwischen den Proteinen von Interesse, weswegen kinetische Studien der lichtkontrollierten Protein-Protein Interaktion durchgeführt werden sollen.

Neben der lichtgesteuerten Interaktion von *AtPhyB* ist wenig über die Interaktionspartner anderer Phytochrome aus *A. thaliana* bekannt. Ein orthogonales Interaktionspaar, das keine Überschneidung zur *AtPhyB*-PIF Interaktion zeigt, wäre von großem Interesse für die Forschung und Anwendung in der Optogenetik. Aus diesem Grund soll innerhalb einer Kooperation ein *AtPhyA* spezifisches Partnerprotein identifiziert und anschließend charakterisiert werden, das lichtabhängig Interaktion mit diesem Phytochrom eingeht.

Ein Nachteil pflanzlicher Phytochrome bei der Anwendung in Säugerzellen im Vergleich zu anderen Photorezeptorsystemen ist die Notwendigkeit der Zugabe von spezifischen Chromophoren. Ein weiteres Ziel war es daher zu verstehen, warum pflanzliche Phytochrome im Gegensatz zu bakteriellen Phytochromen kein Biliverdin verwenden und einen Weg zu finden diese Phytochrome zugänglicher für den Einbau von Biliverdin zu gestalten.

Des Weiteren ist bis heute nicht geklärt, wie das *D. radiodurans* Bakteriophytochrom auf molekularer Ebene seine biologische Funktion vermittelt. Die Erkenntnisse und Techniken, die zur Untersuchung von pflanzlichen Phytochromen eingesetzt werden, sollen daher im Folgenden auf dieses bakterielle Phytochrom angewandt werden.

2 Synopsis

Die vorliegende kumulative Dissertation beinhaltet fünf wissenschaftliche Manuskripte. Von diesen Manuskripten ist eines bereits veröffentlicht, zwei weitere wurden eingereicht und eines dieser beiden befindet sich aktuell in Revision. Bei den anderen beiden Manuskripten handelt es sich zum jetzigen Zeitpunkt um noch nicht eingereichte wissenschaftliche Arbeiten. Der Inhalt aller Manuskripte befasst sich mit der Aufklärung, Charakterisierung und Anwendung der biochemischen bzw. biophysikalischen Eigenschaften von Phytochromen. Die ersten beiden Arbeiten befassen sich dabei mit der lichtkontrollierten Interaktion zwischen dem pflanzlichen Phytochrom B aus *A. thaliana* und dessen Interaktionsfaktoren (Kapitel 2.1 und 2.2). Außerdem wird gezeigt, dass Phytochrom B gegensätzlich zu bisherigen Annahmen Biliverdin als Chromophor verwenden kann und dabei seine Funktionalität erhalten bleibt (Kapitel 2.3). Des Weiteren wurde für die kombinierte, orthogonale Anwendung von pflanzlichen Phytochromen ein spezifischer Interaktionspartner für Phytochrom A charakterisiert (Kapitel 2.4). Neben pflanzlichen Phytochromen wurde auch die biochemische Aktivität zweier bakterieller Phytochrome aufgeklärt und die Interaktion dieser mit ihren jeweiligen Antwortregulatoren untersucht (Kapitel 2.5).

2.1 Funktionelle Charakterisierung der *AtPhyB*-PIF Interaktion

Die erste Veröffentlichung (Kapitel 5.1) behandelt die Charakterisierung der Interaktion zwischen den pflanzlichen Phytochrom B und dessen Interaktionsfaktoren aus *A. thaliana*. Obwohl dieses Interaktionssystem für das Überleben von Pflanzen unentbehrlich ist und auf vielfältige Art in der Optogenetik und Biotechnologie eingesetzt wird, ist überraschend wenig über die tatsächlichen Interaktionsstärken von *AtPhyB* mit den unterschiedlichen PIF Proteinen bekannt. Für diese Interaktion wird lediglich die NTE und das photosensorische Modul (PSM) von *AtPhyB* benötigt (Levskaya et al. 2009). Zusätzlich wurde gezeigt, dass das für die Interaktion mit dem *AtPhyB* benötigte APB Motiv in den ersten 100 Aminosäuren der PIFs codiert ist (Khanna et al. 2004). Dieses Motiv enthält zwei unterschiedlich stark konservierte Bereiche, im Folgenden als APB.A und APB.B bezeichnet. Der APB.A Teil enthält eine E-L-x-x-x-x-G-Q Aminosäurenabfolge, die für die Interaktion unerlässlich ist. Abgesehen von den A- und B-Bereichen weist das APB Motiv kaum Konservierung auf. Innerhalb dieser Arbeit sollte daher das APB Motiv näher charakterisiert werden. Um dieses Ziel zu erreichen, wurden verkürzte und modifizierte APB Konstrukte der meistverwendeten Varianten PIF3 und PIF6 erstellt (Abbildung 15).

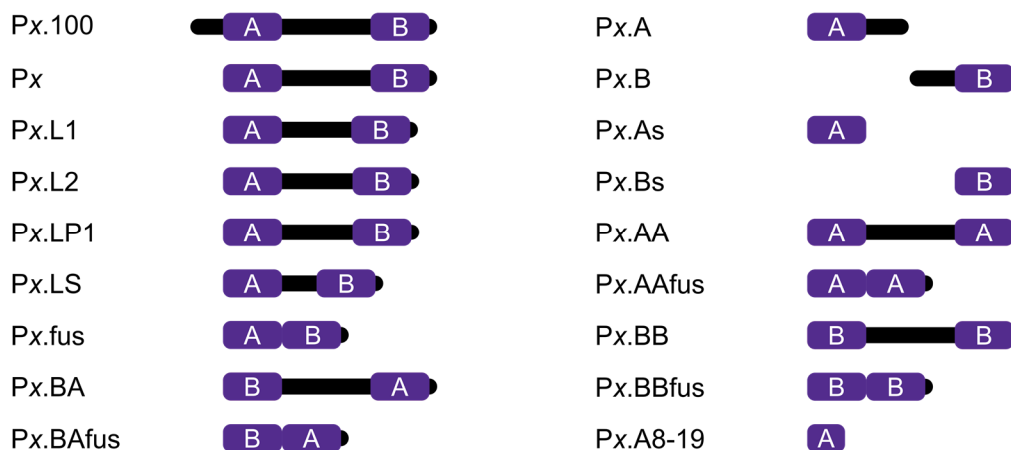


Abbildung 15: Schematische Übersicht der untersuchten APB Varianten. x repräsentiert das jeweils verwendeten PIF3 bzw. PIF6. A bzw. B steht für den jeweiligen APB.A bzw. APB.B Teil. Px.100 entspricht dem vollständigen APB Motiv; Px ist das Motiv ohne die ersten nicht konservierten Aminosäuren; L1, L2, LP1 und LS repräsentieren verkürzte und modifizierte Linker Konstrukte; fus, As bzw. Bs repräsentieren Konstrukte ohne Linker und A8-19 steht für verkürzte A-Varianten.

Die PIF Varianten wurden in *E. coli* exprimiert und die Interaktion der PIFs im Lysats mit gereinigten *AtPhyB*-PSM zunächst anhand des verlangsamen Einflusses auf die thermale Reversion des Phytochroms getestet (Abbildung 16a). Zur Kontrolle der erfolgreichen Expression und zur Quantifizierung wurden die PIF Konstrukte mit einem *enhanced yellow fluorescent protein* (EYFP) Label versehen. Zu Beginn stellte sich die Frage, wie wichtig die nicht konservierten Bereiche für die Interaktion sind. Es zeigte sich, dass die Entfernung N-terminaler Aminosäuren vor dem APB.A Teil die Interaktion mit dem Pfr Zustand nicht negativ beeinflusst (Abbildung 16b). Varianten mit verkürzten und modifizierten Linkern zwischen dem APB.A- und APB.B Teil weisen ebenfalls unverändert Interaktion auf. Überraschenderweise störte auch die vollständige Entfernung des Linkers das System nicht. Um zu testen, ob eine spezifische Topologie des APB Motives für eine Interaktion mit *AtPhyB*-PSM notwendig ist, wurden Konstrukte mit vertauschter Anordnung von APB.A und APB.B Teil erstellt. Auch diese modifizierten PIFs zeigten unverändert Interaktion. Die Ergebnisse zeigen, dass nur die konservierten APB.A und APB.B Bereiche notwendig für das Zusammenspiel mit *AtPhyB*-PSM sind. Daraus ergab sich jedoch die Frage, ob beide Teile gleichermaßen beteiligt sind oder ob nur ein Teil benötigt wird. Die Trennung der beiden Bereiche voneinander ergab, dass lediglich der APB.A Teil mit ca. 25 Aminosäuren für die Interaktion mit *AtPhyB*-PSM unentbehrlich ist (Abbildung 17). Dies wird auch bei Konstrukten mit verdoppelten A- oder B-Teil ersichtlich, die gleiches Verhalten wie die entsprechenden A- und B-Konstrukte zeigten. Eine weitere Reduzierung der Größe des A-Teils war nicht möglich.

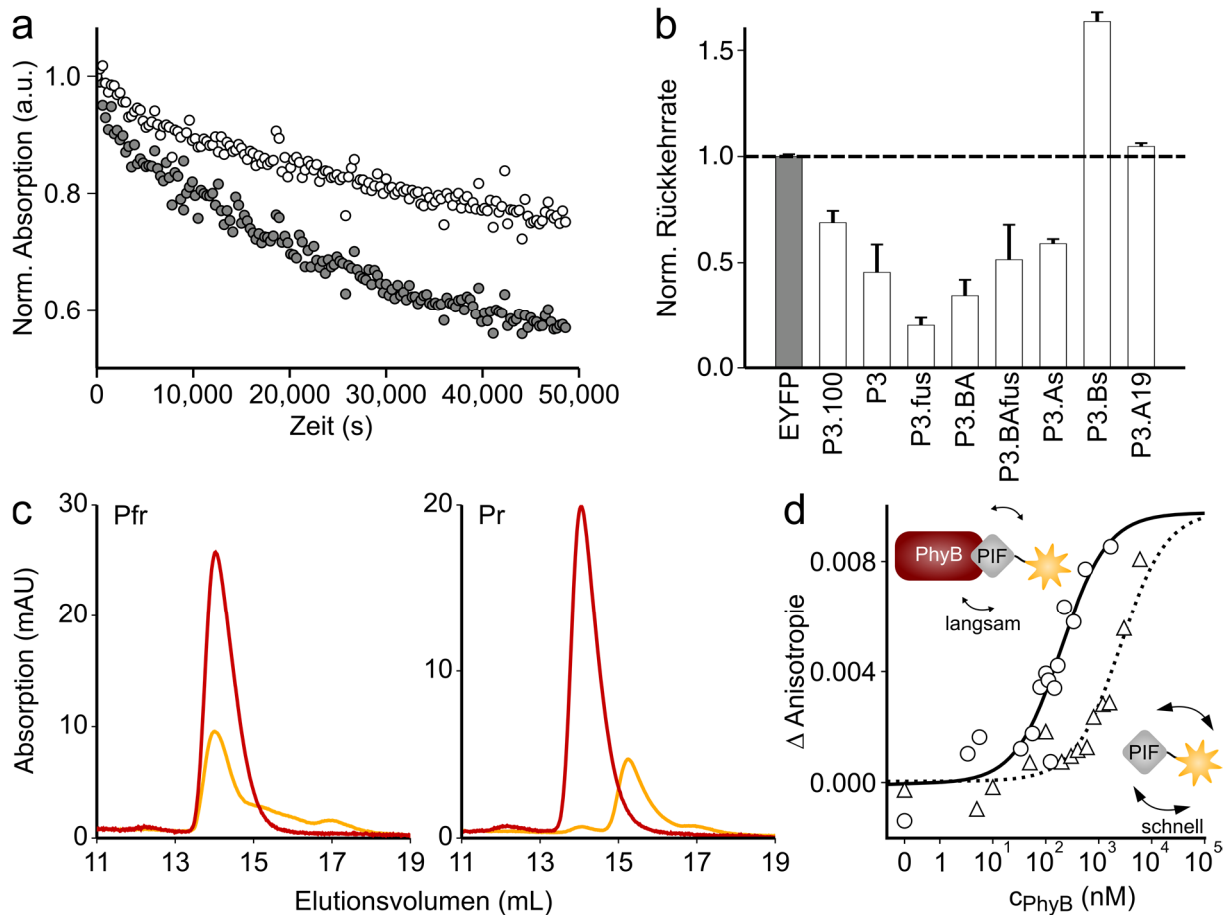


Abbildung 16: Biochemische Analyse der *AtPhyB*-PIF Interaktion. (a) Normierte Absorption von *AtPhyB*-PSM bei 720 nm nach vorheriger Bestrahlung mit Rotlicht in Anwesenheit von P3.100-EYFP (weiß) oder EYFP (grau). (b) Normiert initiale Rückkehraten für *AtPhyB*-PSM in Anwesenheit von EYFP oder verschiedenen PIF-EYFP Konstrukten. (c) Größenausschlusschromatographie von *AtPhyB*-PSM und P3.100-EYFP Gemischen nach Bestrahlung mit Rotlicht (Pfr) oder Fernrotlicht (Pr). Absorptionsmessung erfolgte bei 514 nm (gelb) und 650 nm (rot). (d) Änderung der Fluoreszenzanisotropie von P3.100-EYFP in Anwesenheit von *AtPhyB*-PSM. Nach Bestrahlung mit Rotlicht (Kreise) liegt *AtPhyB*-PSM im Pfr Zustand vor und interagiert mit P3.100-EYFP. Nach Fernrotlichtbestrahlung (Dreiecke) geht ein Großteil der Interaktion verloren.

Zur Bestätigung der Ergebnisse und um zu ermitteln ob die Interaktion der PIF Varianten mit *AtPhyB*-PSM noch in Abhängigkeit der Lichtbedingungen erfolgt, wurde Größenausschlusschromatographie mit gereinigten PIF-EYFP Konstrukten und *AtPhyB*-PSM nach vorheriger Bestrahlung mit Rot- bzw. Fernrotlicht durchgeführt (Abbildung 16c). Alle Varianten, die verlangsamen den Einfluss auf die thermale Reversion von *AtPhyB*-PSM zeigten, demonstrierten auch während der Größenausschlusschromatographie eine 1:1 Interaktion mit dem Pfr Zustand des Phytochroms. Eine vorherige Bestrahlung mit Fernrotlicht versetzte *AtPhyB*-PSM in den Pr Zustand und die Interaktion mit den PIF Konstrukten ging verloren (Abbildung 16c). Dies zeigt, dass die Wechselwirkung bei allen interagierenden PIF Varianten

noch lichtabhängig erfolgt. Die Größenausschlusschromatographie ermöglicht es auch den Oligomerisierungszustand der PIF Konstrukte zu bestimmen. PIFs agieren in der Natur als *bHLH*-Transkriptionsfaktoren und beeinflussen Genexpression entweder als Homo- oder Heterodimere (Castillon et al. 2007; Pham et al. 2018). Der homodimerische Charakter findet sich ebenfalls im APB Motiv wieder und ist nicht im monomeren EYFP Label begründet. Alle Varianten ohne APB.A Teil liegen monomerisch vor, was auf eine wichtige Rolle des A-Teils bei der Ausbildung von Dimeren hindeutet. Der A-Teil scheint aber allein nicht ausreichend für die Bildung von Dimeren zu sein, da Varianten nur mit A-Teil ohne Linker-Aminosäuren monomerisch vorliegen (Abbildung 17).

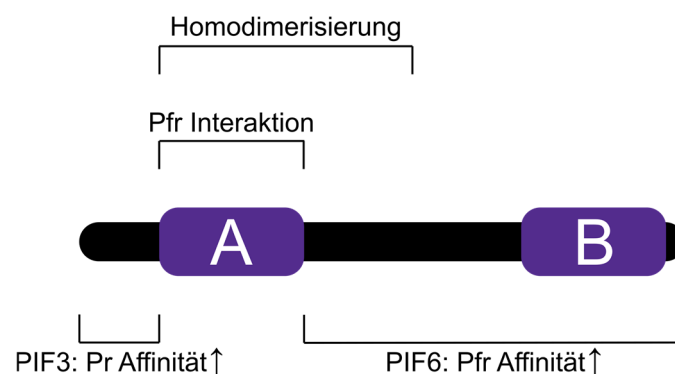


Abbildung 17: Schematische Übersicht der neuen Erkenntnisse für das APB Motiv. Der APB.A Teil (A) ist unentbehrlich für die lichtabhängige Interaktion mit dem Pfr Zustand von *AtPhyB*. Die N-terminalen, nicht konservierten Aminosäuren erhöhen bei PIF3 die Interaktionsstärke mit dem Pr Zustand. Der APB.B Teil (B) von PIF6 erhöht im Zusammenspiel mit dem A-Teil die Affinität für den Pfr Zustand von *AtPhyB* bei ausreichender Linkerlänge zwischen den beiden Teilen. Für die Homodimerisierung des APB Motivs ist der A-Teil zusammen mit einigen Aminosäuren des Linkers verantwortlich.

Da bisheriger Methoden lediglich qualitative Aussagen bezüglich des Zusammenspiels zwischen *AtPhyB*-PSM und PIFs lieferten, war es von Interesse zu bestimmen welche Interaktionsstärke das APB Motiv und modifizierte Varianten mit dem Phytochrom besitzen. Zu diesem Zweck wurde Fluoreszenzanisotropie angewandt und die Drehbewegung des EYFP Labels an den PIF Proteinen in Lösung betrachtet. In einem Gemisch aus *AtPhyB*-PSM im Pr Zustand und PIF-EYFP liegt das PIF Konstrukt ungebunden vor, wodurch es in seiner Beweglichkeit uneingeschränkt ist. Kommt es nach Rotlichtbestrahlung zur Interaktion mit *AtPhyB*-PSM erhöht sich die Gesamtgröße durch Ausbildung von *AtPhyB*-PIF Heterodimeren. Diese Größenänderung hat eine Erhöhung des hydrodynamischen Radius und dadurch Reduzierung der Drehbeweglichkeit des EYFP Labels zur Folge. Bei konstanter PIF-EYFP und zunehmender *AtPhyB*-PSM Konzentration können somit Bindeisothermen aufgezeichnet

werden, aus denen sich die Dissoziationskonstante K_D ermitteln lassen (Abbildung 16d). Abhängig vom Belichtungszustand und der Menge des gebildeten Pfr-Anteils können somit Interaktionsstärken für den Pr und Pfr Zustand bestimmt werden. Der Vergleich der K_D Werte der unveränderten APB Motive ergab eine deutlich höhere Affinität von PIF6 ($K_D = 10 \text{ nM} \pm 8 \text{ nM}$) für den Pfr Zustand von *AtPhyB*-PSM als dies im direkten Vergleich mit PIF3 ($K_D = 200 \text{ nM} \pm 70 \text{ nM}$) der Fall ist. Während für PIF6 keine Interaktion mit dem Pr Zustand des *AtPhyB* festgestellt wurde, konnte für PIF3 noch schwache Affinität extrapoliert werden ($K_D > 2 \text{ } \mu\text{M}$). Die unterschiedlichen Affinitäten der beiden PIFs, sowohl für den Pr als auch den Pfr Zustand, ist vermutlich in ihrer unterschiedlichen biologischen Funktion begründet. Während PIF3, wie der Großteil der anderen PIFs, als negativer Regulator für die Photomorphogenese agiert (Pham et al. 2018), scheint PIF6 als positiver Regulator zu wirken, da gezeigt werden konnte, dass eine Überexpression von PIF6 Hypokotylwachstum unter Rotlichteinfluss inhibiert (Penfield et al. 2010). Der ausgeprägte Unterschied zwischen Pr und Pfr Interaktion bei PIF6 scheint daher ggf. dabei zu helfen vorzeitige Inhibierung des Hypokotylwachstums zu verhindern. Interessanterweise führt die Abtrennung der nicht konservierten, N-terminalen Aminosäuren vor dem APB.A Teil im Falle des PIF3 dazu, dass dessen Affinität zum Pr Zustand des *AtPhyB*-PSM sinkt ($K_D > 10 \text{ } \mu\text{M}$) was für eine Aufgabe dieser Aminosäurereste bei der Feinregulierung der basalen Aktivität spricht (Abbildung 17). Im Gegensatz dazu hat die Entfernung des Linkers oder des B-Teils kaum Einfluss auf die Interaktionsstärken von PIF3, aber reduziert die Pfr-Interaktion von PIF6 und *AtPhyB*-PSM deutlich (Abbildung 17). Bei PIF6 scheint also der B-Teil bei der Regulierung der Interaktionsstärke mit dem Pfr-Anteil beteiligt zu sein. Als Resultat weisen alle Varianten bei denen Linker oder B-Teil entfernt wurden kaum noch Unterschiede zwischen PIF3 und PIF6 bezüglich ihrer Interaktion mit *AtPhyB*-PSM auf. Zusammenfassend kann also geschlussfolgert werden, dass die nicht konservierten Aminosäuren des APB Motivs bei der Feinregulierung der *AtPhyB*-PIF Interaktion beteiligt sind und der B-Teil zumindest bei PIF6 einen Einfluss auf die Pfr-Interaktion hat. Die erhaltenden Erkenntnisse stellen somit einen weiteren Beitrag zum besseren Verständnis des Zusammenspiels zwischen Rotlicht- und Fernrotlichtinteraktion, der daraus resultierenden Konkurrenz verschiedener PIFs um DNA-Bindestellen und des durch Phosphorylierung vermittelten Proteinabbau von PIFs dar (Pham et al. 2018; Ni et al. 2017).

Die in dieser Arbeit erzeugten PIF Varianten haben aber nicht nur Relevanz für den biologischen Kontext, sondern auch für die Anwendung in der Optogenetik. Durch den Einsatz der neuen PIFs zur lichtabhängigen Kontrolle der *secreted alkaline phosphatase* (SEAP)

Reportergenexpression konnten die Resultate der biochemischen Charakterisierung bestätigt werden (Abbildung 18a). Konstrukte, die den vollständigen APB.A Teil besitzen zeigen auch innerhalb der Anwendung in Zellkultur lichtkontrollierte Reportergenexpression, wohingegen der APB.B Teil allein oder verkürzte APB.A Varianten keine Genexpression mehr auslösen. Die erhaltenen Erkenntnisse konnten ebenfalls auf PIF1 Konstrukte übertragen werden, was die Relevanz der Ergebnisse verdeutlicht. Außerdem wurden die Konstrukte erfolgreich zur lichtschtbaren Membranlokalisierung eingesetzt (Abbildung 18b). Die erzeugten PIF Varianten stellen daher aufgrund ihrer unterschiedlichen Interaktionsstärken eine Erweiterung des *AtPhyB*-PIF Systems dar.

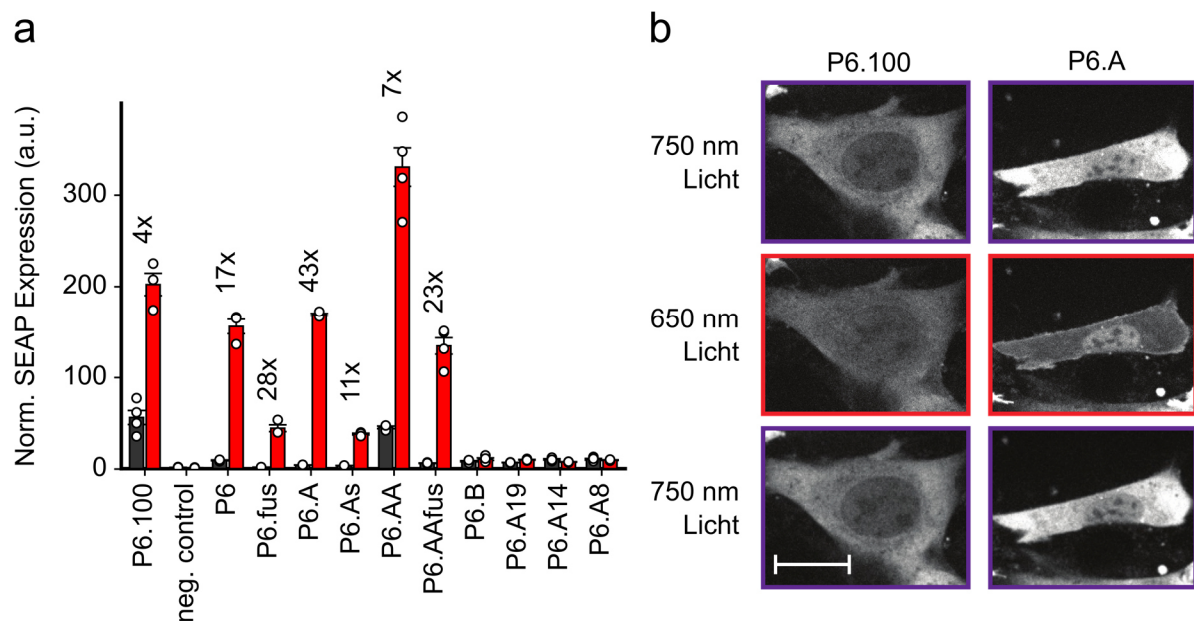


Abbildung 18: Optogenetische Anwendung der *AtPhyB*-PIF Interaktion. (a) Normierte *secreted alkaline phosphatase* (SEAP) Expression unter Kontrolle der lichtabhängigen Interaktion von *AtPhyB*-PSM und PIF6 Konstrukten in Dunkelheit (schwarz) oder unter Rotlicht (rot). Experimente durchgeführt von der Arbeitsgruppe Zurbriggen. (b) Fluoreszenzmikroskopieaufnahmen von NIH-3T3 Zellen die *AtPhyB*-PSM-CAAX und unterschiedliche EYFP-PIF6 Varianten exprimiert haben. Der Größenmaßstab entspricht 20 μ m. Die Lichtschaltbarkeit des Systems konnte durch abwechselnde Bestrahlung mit Rotlicht (rot) oder Fernrotlicht (lila) gezeigt werden. Experimente durchgeführt von der Arbeitsgruppe Toettcher.

2.2 Biophysikalische Untersuchung der *AtPhyB*-PIF Interaktion

Das zweite Manuskript (Kapitel 5.2) beschäftigt sich mit einer detaillierten Charakterisierung der *AtPhyB*-PIF Interaktion mit Hilfe von fluoreszenzspektroskopischen Verfahren. Zu diesem Zweck wurde das monomerische P6.A, das innerhalb der ersten Veröffentlichung (Kapitel 5.1) generiert wurde, mit einer optimierten Version des roten Fluoreszenzproteins aus *Discosoma*

sp. (*DsRed*) fusioniert (Strack et al. 2008). Das resultierende P6.A-*DsRed* ist ein tetramerisches Konstrukt, das lichtabhängige Interaktion mit *AtPhyB*-PSM eingeht (Abbildung 19a). Multivalente, lichtabhängige PPIs sind von hohem Interesse für die Erforschung von physiologischen Prozessen wie der durch Assemblierung vermittelten Rezeptoraktivierung oder der Ausbildung bzw. Auflösung subzellulärer Kompartimente (Shin et al. 2017; Dine et al. 2018).

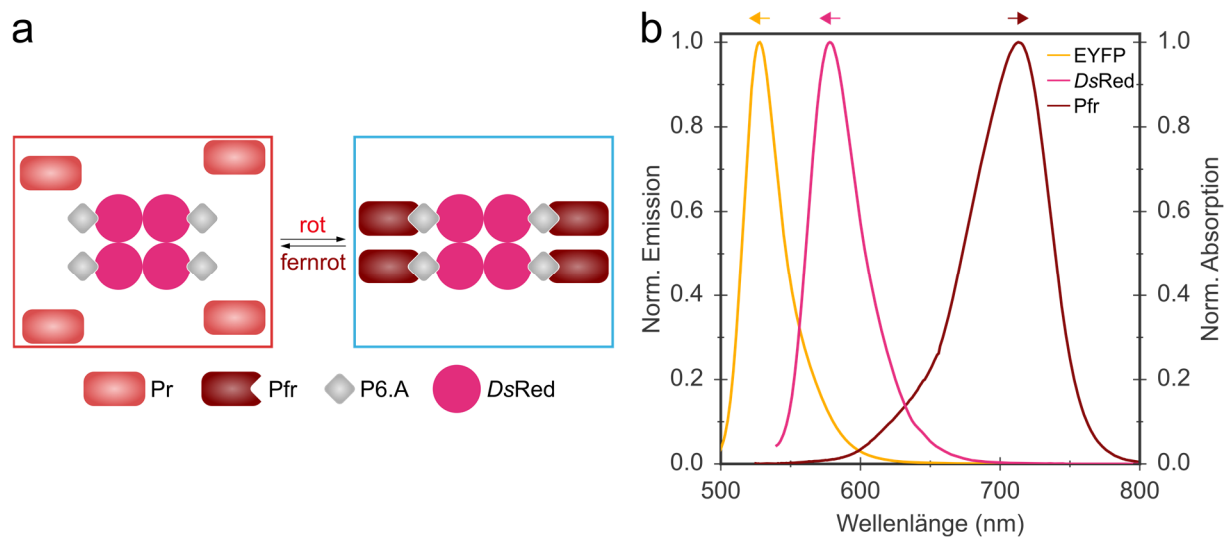


Abbildung 19: Anwendung von P6.A-*DsRed* zur Untersuchung der lichtschtbaren Interaktion von *AtPhyB* und PIF Proteinen. (a) Schematische Darstellung der Interaktion des tetramerischen P6.A-*DsRed*s und *AtPhyB*-PSM im Pr oder Pfr Zustand. (b) Normierte Fluoreszenzemissionsspektren von EYFP und *DsRed* und normiertes Absorptionsspektrum des reinen Pfr Zustands von *AtPhyB*-PSM.

Im Gegensatz zum zuvor verwendeten EYFP besitzt *DsRed* ein weiter rotverschobenes Emissionsspektrum, welches einen besseren Förster-Resonanzenergietransfer (FRET) zum interagierenden Pfr-Zustand von *AtPhyB*-PSM ermöglicht (Abbildung 19b). Wenn Interaktion zwischen P6.A-*DsRed* und *AtPhyB*-PSM stattfindet, nähern sich *DsRed* und *AtPhyB*, wodurch ein Teil der Anregungsenergie von *DsRed* auf das *AtPhyB* übertragen wird. Da die Fluoreszenz von *AtPhyB*-PSM jedoch ohne das Einbringen entsprechender Mutationen im Vergleich zu optimierten Fluoreszenzproteinen nur schwach ist, wurde die Abnahme der Fluoreszenzintensität von *DsRed* als Maß für die Interaktion herangezogen. Auf die Modifikation von *AtPhyB*-PSM zur Steigerung der Fluoreszenz wurde aufgrund des daraus resultierenden gestörten Pr → Pfr Übergangs verzichtet (Su und Lagarias 2007). Bei gleichbleibender P6.A-*DsRed* und zunehmender *AtPhyB*-PSM Konzentration kann somit anhand der Abnahme der absoluten Fluoreszenzintensität des *DsRed* eine Bindungskurve für die P6.A-*AtPhyB* Interaktion erstellt werden (Abbildung 20a). Die erhaltene

Dissoziationskonstante K_D beträgt (50 ± 15) nM. Da bei dieser Methode der Fluoreszenzmissionsunterschied zwischen freiem und gebundenem P6.A-*DsRed* bestimmt wird, kann die resultierende Bindekurve nur Auskunft über die Interaktion von P6.A mit dem Pfr-Zustand von *AtPhyB* geben.

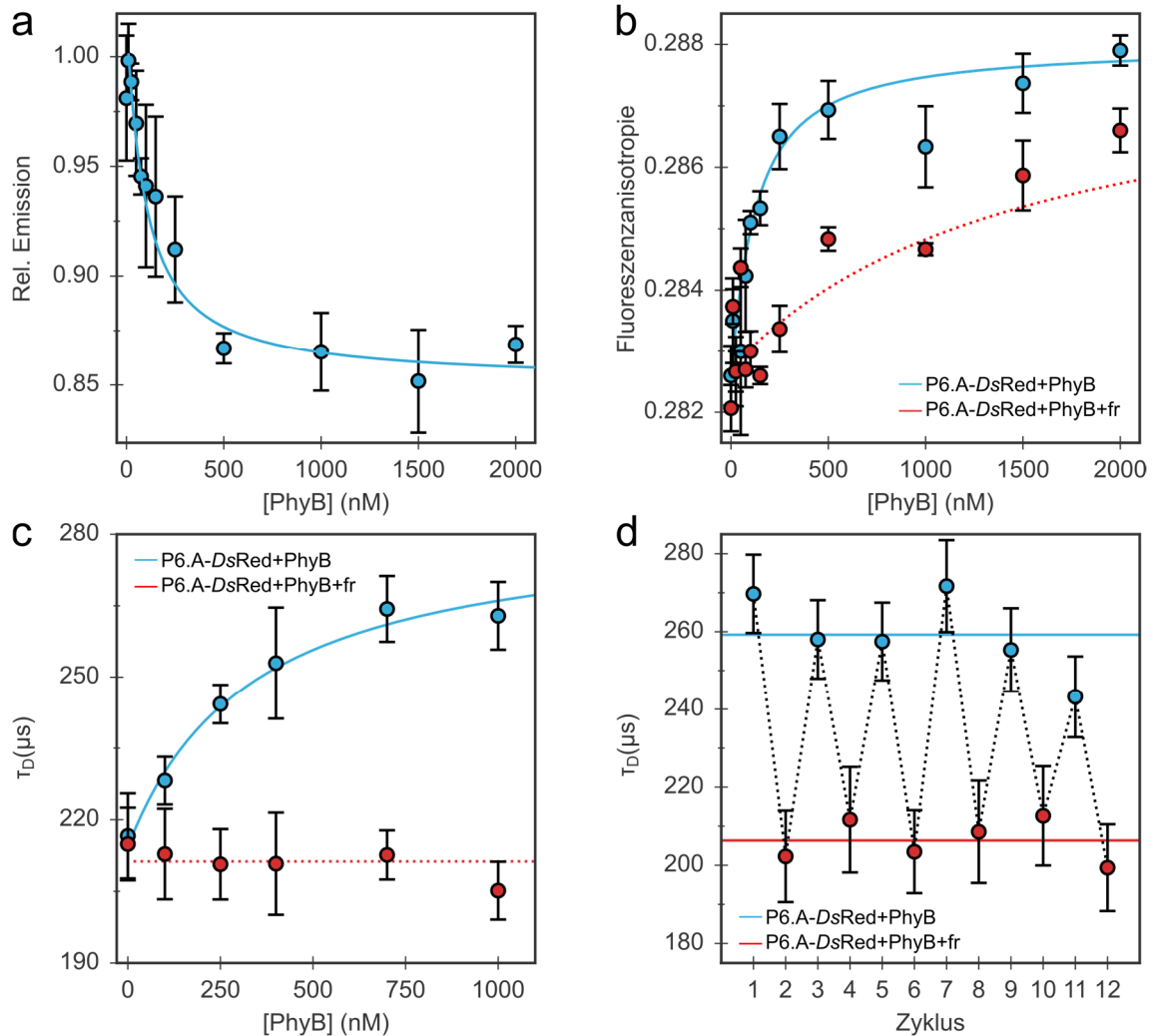


Abbildung 20: Interaktionsstudien zwischen P6.A-*DsRed* und *AtPhyB*-PSM (PhyB). (a) Untersuchung der P6.A-*AtPhyB* Interaktion anhand der relativen Fluoreszenzintensitätsänderung des *DsRed* Fluorophors aufgrund von Förster-Resonanzenergietransfer (FRET). (b) Fluoreszenzanisotropiemessung von P6.A-*DsRed* zur Bestimmung der Interaktionsstärke mit dem Pr (rot) und Pfr (blau) Zustand von PhyB nach vorheriger Bestrahlung mit Rot- oder Fernrotlicht (fr). (c) Fluoreszenzkorrelationsspektroskopiemessung von P6.A-*DsRed* zur Bestimmung der Interaktionsstärke von P6.A mit dem Pr und Pfr Zustand von PhyB nach Rotlicht Beleuchtung oder bei konstanter Fernrotlichtbeleuchtung. (d) Fluoreszenzkorrelationsspektroskopiemessung einer P6.A-*DsRed* Probe in Anwesenheit von PhyB nach Rotlichtbestrahlung (ungerade Zyklenzahl) und während Fernrotlichtbestrahlung (gerade Zyklenzahl).

Aus diesem Grund wurde Fluoreszenzanisotropie verwendet, um Bindekurven für die Pr- und Pfr-Interaktion zu erhalten (Abbildung 20b). Bindung zwischen P6.A-*DsRed* und *AtPhyB*-PSM

bewirkt eine Vergrößerung des hydrodynamischen Radius und damit eine Verlangsamung der Drehbeweglichkeit des *DsRed* Fluorophors. Die Änderung der Fluoreszenzanisotropie von P6.A-*DsRed* bei steigender *AtPhyB*-PSM Konzentration konnte, analog zu vorherigen Messungen mit P6.A-EYFP, durch eine einfache Bindeisotherme beschrieben werden. Mit Hilfe der Fluoreszenzanisotropie wurde eine Bindestärke für P6.A mit dem Pfr-Zustand von *AtPhyB*-PSM von (50 ± 10) nM nach vorheriger Beleuchtung mit Rotlicht bestimmt. Für die Pr-Interaktion nach Fernrotlichtbeleuchtung wurde ein $K_D > 1 \mu\text{M}$ extrapoliert. Die Werte sind in vergleichbarer Größenordnung zu den zuvor für P6.A-EYFP mittels Fluoreszenzanisotropie erhaltenen K_D Werten von (280 ± 100) nM für die Pfr-Interaktion und $> 2 \mu\text{M}$ für die Pr-Interaktion. Des Weiteren wurde die Stöchiometrie des verwendeten Systems mit Hilfe von Fluoreszenzanisotropiemessungen, bei denen P6.A-*DsRed* in Konzentrationen über dem K_D eingesetzt wurde, bestimmt. Liegt mehr P6.A-*DsRed* als *AtPhyB*-PSM vor bindet jeweils ein P6.A-Tetramer ein *AtPhyB*-PSM Molekül. Im deutlichen Überschuss von *AtPhyB*-PSM binden jeweils vier Moleküle an das P6.A-*DsRed*. Da sich die Fluoreszenzanisotropie bei der Bestimmung der K_D Werte durch ein einfaches Bindemodell beschreiben lässt und weil die Stöchiometrie auf vier frei zugängliche Bindestellen hindeutet, ist davon auszugehen, dass sich die vier Bindestellen des P6.A-*DsRed* unabhängig zueinander verhalten und die Tetramerisierung keinen wesentlichen Einfluss auf die *AtPhyB*-PIF Interaktion hat.

Um die Interaktion auf der molekularen Ebene besser zu verstehen, wurde anschließend Fluoreszenzkorrelationsspektroskopie angewandt. Bei dieser Methode wird ein Fluorophor in einem konfokalen, Femtoliter großen Volumen mit Hilfe eines Lasers angeregt und der zeitliche Verlauf des Emissionssignals gemessen. Das Messsignal fluktuiert dabei abhängig vom Diffusionsverhalten der fluoreszierenden Moleküle, da die Teilchen in Abhängigkeit ihrer Größe das Fokalkvolumen schneller oder langsamer durchqueren. Durch Anwendung der Autokorrelationsfunktion kann die Diffusionszeit des betrachteten Fluorophoren bestimmt werden. Bei geeigneter Verdünnung sind somit sogar Einzelmolekülmessungen möglich. Der Versuchsaufbau dieser Methode gestattet es die Probe während des Messvorgangs zusätzlich zum Anregungslaserlicht auch mit Fernrotlicht zu bestrahlen. Die erhaltenen Diffusionszeiten bei konstanter P6.A-*DsRed* Konzentration und steigender *AtPhyB*-PSM Konzentration beschreiben ähnlich wie bei vorherigen Messungen ebenfalls den Verlauf einer einfachen Bindekurve, wenn sich das Phytochrom im Pfr Zustand befindet (Abbildung 20c). Die erhaltene Interaktionsstärke beträgt (180 ± 60) nM und ist in guter Übereinstimmung mit vorherigen Messungen. Die parallele Bestrahlung mit Fernrotlicht bewirkt, dass keine Interaktion mit dem

Pr Zustand mehr festgestellt werden kann (Abbildung 20c). Der Grund hierfür ist vermutlich darin begründet, dass die konstante Beleuchtung mit Fernrotlicht auch geringste Restmengen an Pfr sofort nach Bildung wieder in den Pr Zustand überführt und dadurch unspezifische Interaktion verhindert wird. Bei der Fluoreszenzanisotropiemessung wird die Probe vor dem eigentlichen Messvorgang mit Fernrotlicht beleuchtet. Während der Messung wird demnach die Interaktion des PIFs mit dem durch Fernrotlicht gebildeten Gleichgewicht aus Pr und Pfr Zustand vermessen. Geringfügige Restmengen an Pfr können daher Einfluss auf die Pr Interaktion haben. Die Fluoreszenzkorrelationsmessungen verdeutlicht daher, dass unter direktem Lichteinfluss der Interaktionsunterschied zwischen Pr und Pfr Interaktion sogar noch ausgeprägter ist als zuvor vermutet. Zusätzlich wurde die Robustheit der *AtPhyB*-PIF Interaktion verdeutlicht, indem das System mehrere Male zwischen dem interagierten und nicht interagierenden Zustand hin und her geschaltet wurde (Abbildung 20d). Die Ergebnisse zeigen, dass die Interaktion mehrmals aktiviert und inaktiviert werden kann ohne, dass ein Verlust der Effizienz beobachtet wird. Dies stellt einen entscheidenden Vorteil im Vergleich zu chemisch induzierten Interaktionssystemen dar.

Neben der Interaktionsstärke ist auch die Dynamik eines Interaktionssystems von Interesse. Zur Bestimmung dieser Parameter wurde erneut der FRET Effekt zwischen *DsRed* und *AtPhyB*-PSM ausgenutzt. Zeichnet man das Fluoreszenzsignal von P6.A-*DsRed* in Anwesenheit von sehr hohen Mengen *AtPhyB*-PSM auf und beleuchtet die Probe zeitgleich mit Rot- oder Fernrotlicht, dann kann die Änderung im Interaktionsverhalten anhand der Änderung des Fluoreszenzsignals über die Zeit bestimmt werden (Abbildung 21a). Bestimmt man diese Assoziations- und Dissoziationskinetiken bei verschiedenen *AtPhyB*-PSM Konzentrationen lassen sich Zusammenhänge zwischen der *AtPhyB*-PSM Konzentration und der Dynamik des Systems bestimmen (Abbildung 21b). Aus der Steigung und dem Achsenabschnitt der Auftragung der beobachteten kinetischen Parameter gegen die eingesetzte *AtPhyB*-PSM Konzentration lassen sich die Assoziations- und Dissoziationsraten k_1 und k_{-1} der *AtPhyB*-PIF Interaktion für den Pr und Pfr Zustand bestimmen. Während für die beobachteten Raten beim Pfr Zustand von *AtPhyB*-PSM eine ausgeprägte Abhängigkeit von der Konzentration beobachtet wurde, ist dies beim Pr Zustand nicht der Fall. So wurde für den Pfr Zustand $k_1 = (3.9 \pm 0.5) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ und $k_{-1} = (0.42 \pm 0.06) \text{ s}^{-1}$ bestimmt, während eine verlässliche Bestimmung des $k_1 [(0.8 \pm 5.0) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}]$ für den Pr Zustand nicht möglich war. k_{-1} im Pr Zustand beträgt $(0.19 \pm 0.09) \text{ s}^{-1}$. Die gemessenen Assoziations- und Dissoziationsraten verdeutlichen, dass die ausgeprägte Interaktionsstärke zwischen PIFs und dem Pfr-Zustand von

AtPhyB in der Assoziation begründet liegen. Dies ist eine überraschende Entdeckung, da starke Protein-Ligand Interaktion in der Regel das Resultat langsamer Dissoziationsreaktionen sind (Schreiber et al. 2009). Die hier durchgeführte Untersuchung liefert somit neue Erkenntnisse bezüglich der zuvor unbekannten kinetischen Parameter der *AtPhyB*-PIF Interaktion.

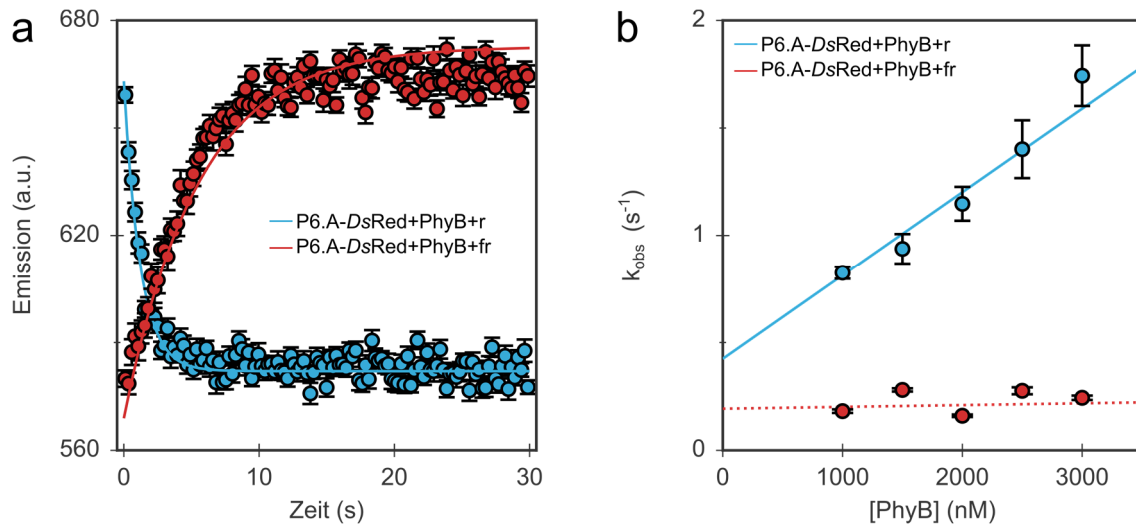


Abbildung 21: Fluoreszenzintensitätsmessungen zur Bestimmung der Dynamik der Interaktion zwischen P6.A-DsRed und *AtPhyB*-PSM (PhyB). (a) Zeitlicher Verlauf der Fluoreszenzintensität von P6.A-DsRed in Anwesenheit von 1000 nM PhyB nach Aktivierung von Rot- (r) bzw. Fernrotlichtbestrahlung (fr). (b) Auftragung der beobachteten Kinetischen Parameter k_{obs} für die Assoziation (blau) und Dissoziation (rot) gegen die PhyB Konzentration.

2.3 Biliverdin als Chromophor in pflanzlichen Phytochromen

Innerhalb der dritten Arbeit (Kapitel 5.3) wird der funktionelle Einbau von Biliverdin (BV) als Chromophor in *AtPhyB* untersucht. Im Gegensatz zu früheren Annahmen, dass pflanzliche Phytochrome den Bilinchromophor BV nicht oder im besten Fall nur extrem schlecht inkorporieren können (Li und Lagarias 1992; Rockwell und Lagarias 2010; Spudich und Briggs 2005), wurde hier gezeigt, dass ein funktionaler Einbau von BV in *AtPhyB*-PSM möglich ist. Zu diesem Zweck wurde *AtPhyB*-PSM zusammen mit *Synechocystis sp.* Hämoxygenase 1 (HO1) in *E. coli* Zellen für 16 h und 18 °C koexprimiert. In den ersten Experimenten wurde außerdem ein Inkubationsschritt der Proteinlösung mit Überschuss an freiem BV während der Proteinreinigung durchgeführt, da ein schlechter Chromophoreinbau zu vermuten war. Überraschenderweise zeigte das *AtPhyB*-PSM ohne jegliche Veränderungen an der Aminosäuresequenz bereits einen guten Einbaugrad von 62 %. Im direkten Vergleich war für einen erfolgreichen BV Einbau in cyanobakteriellen Phytochromen das Einbringen diverser Veränderungen innerhalb der Chromophorbindetasche notwendig (Fushimi et al. 2019;

Kuwasaki et al. 2019). Unter den genannten Expressionsbedingungen kann für PCB, einem reduzierten Bilin das bereitwillig von *AtPhyB*-PSM inkorporiert wird, ein Chromophoreinbau von 93 % erzielt werden. Es sei angemerkt, dass zur PCB Produktion das Enzym *pcyA* zusätzlich benötigt und kein Chromophor extern zugegeben wurde. Anschließend wurde versucht durch Verlängerung der Expressionszeit auf 40 h und durch die gleichzeitige Bestrahlung mit 800 nm Fernrotlicht während der Expression den Einbau auch ohne BV Zugabe zu steigern. Dies resultierte schlussendlich in einen Einbaugrad von 84 %. Die verlängerte Expressionszeit steigert den Chromophoreinbau vermutlich, weil bei der Bildung von BV durch HO1 die Abgabe des Chromophors an das Zytosol nur langsam erfolgt. Bevorzugt übertragen Hämoxygenasen das gebildete BV in einem kooperativen Prozess direkt an Oxidoreduktasen anstatt es in die umgebende Matrix abzugeben (Liu und Ortiz de Montellano 2000). Der positive Effekt des Fernrotlichts ist vermutlich darauf zurückzuführen, dass diese Lichtbedingungen die Ausbildung des eigentlich im Dunkeln adaptierten Pr Zustands begünstigen. Konventionelle Phytochrome, darunter *PhyB* aus *A. thaliana* und *BphP* aus *D. radiodurans*, adaptieren den Pr Zustand in Dunkelheit, weswegen vermutet wird, dass der Einbau in dieser Konformation bevorzugt erfolgt (Burgie und Vierstra 2014). Eine Temperaturerhöhung während der Expression durch die Fernrotlichtbestrahlung kann ausgeschlossen werden, da sich die Zellen unter ständiger Temperaturkontrolle befanden.

Der spektroskopische Vergleich zwischen *AtPhyB*-PSM mit PCB oder BV und dem bakteriellen Phytochrom *DrBphP* mit BV zeigt, dass *AtPhyB*-PSM mit BV deutlich ähnlichere Eigenschaften zum *AtPhyB*-PSM mit PCB als zum bakteriellen Phytochrom besitzt (Abbildung 22a-c). Die Maxima für den Pr und Pfr Zustand sind mit ca. 13 nm nur leicht rotverschoben im Vergleich zu *AtPhyB*-PSM mit PCB, jedoch mit ca. 20-40 nm deutlich stärker blauverschoben als bei *DrBphP* mit BV. Auch die thermische Reversion des *AtPhyB*-PSM mit BV ist der des gleichen Proteins mit PCB ähnlicher als der des bakteriellen Phytochroms. Die einzelnen Phasen haben jedoch unterschiedliche Gewichtung in Abhängigkeit vom verwendeten Chromophor, wodurch die thermische Reversion in ihrer Gesamtheit mit BV im *AtPhyB*-PSM schneller erfolgt als es mit PCB im *AtPhyB*-PSM der Fall ist (Abbildung 22d). Um die spektralen Charakteristika noch genauer zu untersuchen, wurde ultraschnelle Absorptionsspektroskopie angewandt. Diese Methode erlaubt es kurzlebige spektroskopische Zustände beim Übergang vom Pr in den Pfr Zustand zu betrachten (Abbildung 9). Die kurzlebigen Absorptionsspektren und das primäre Photoproduktspektrum, Lumi-R, beim bakteriellen Phytochrom sind hier ebenfalls deutlich rotverschoben im Vergleich zu den

Spektren für das *AtPhyB*-PSM. Der Chromophor in *AtPhyB*-PSM kann anhand des Anteils der stimulierten Emission innerhalb der kurzlebigen ($\sim$ ps) Spektren unterschieden werden. Obwohl es aufgrund technischer Limitierungen nicht möglich ist die sehr frühen Spektren ohne Einfluss des Photoproduktspektrums zu messen (Dietzek et al. 2007), deuten die Ergebnisse zusammen mit kürzlichen Beobachtungen während zeitaufgelöste Kristallographie darauf hin, dass die Isomerisierung des Chromophoren innerhalb der ersten Pikosekunden erfolgt (Claesson et al. 2020). Eine überraschende Beobachtung, da bisher für die Ausbildung des Lumi-R eine deutlich längere Zeitspanne von bis zu mehreren hundert Pikosekunden angenommen wurde (Choudry et al. 2018; Ihalaainen et al. 2018; Kennis und Groot 2007).

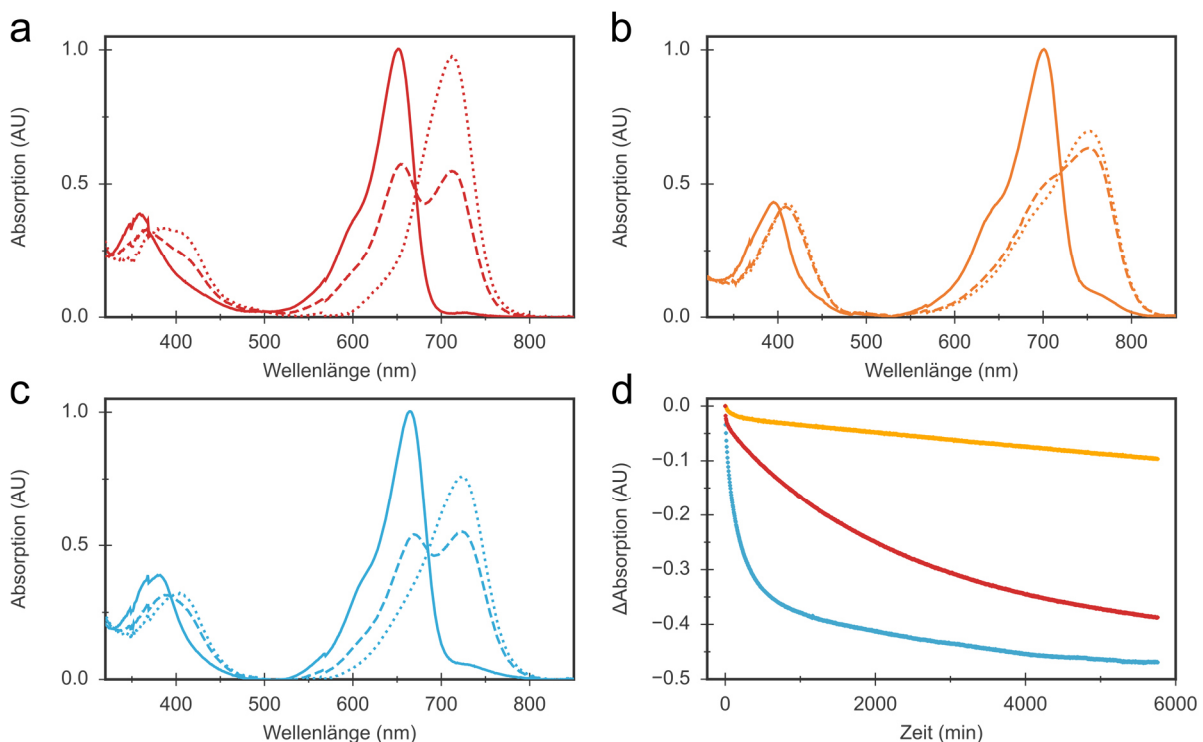


Abbildung 22: Spektrale Charakterisierung des photosensorischen Moduls (PSM) von verschiedenen Phytochromen (a) Absorptionsspektren von *AtPhyB*-PSM mit PCB im Pr Zustand (durchgezogene Linie), Pfr Zustand (gestrichelte Linie) und 100 % Pfr Spektrum (gepunktete Linie) berechnet nach (Butler et al. 1964). (b) wie in (a) aber *DrBphP*-PSM mit BV. (c) wie in (a) aber *AtPhyB*-PSM mit BV. (d) Thermale Reversion von *AtPhyB*-PSM mit PCB (rot) oder BV (blau) und *DrBphP*-PSM mit BV (orange).

Der Vergleich der spektroskopischen Eigenschaften lässt vermuten, dass die spektrale Verschiebung nicht aufgrund der umgebenden Aminosäuren erfolgt. Eine Untersuchung bei der die Aminosäuren in näherer Chromophorumgebung verändert wurden zeigte beispielsweise nur geringen Einfluss auf die Position der Absorptionsmaxima (Wagner et al. 2008). Dem zu Folge wird vermutet, dass ein alternativer Bindemechanismus für BV in *AtPhyB*-PSM die Ursache

für die veränderten spektralen Eigenschaften ist. Bakterielle Phytochrome bilden mit BV eine kovalente Bindung zwischen dem C3² Atom des Chromophoren und einem Cystein in der PAS Domäne (Abbildung 23a). In pflanzlichen Phytochromen erfolgt die Ausbildung der Bindung zu reduzierten Bilinen jedoch zwischen der C3¹ Position und einem Cystein der GAF Domäne (Abbildung 23b). Aufgrund der räumlichen Gegebenheiten innerhalb des Proteins ist daher zu vermuten, dass die Bindung zwischen Cystein und BV in pflanzlichen Phytochromen ebenfalls am C3¹ Atom erfolgt (Abbildung 23c). Im Gegensatz zur Reaktion mit reduzierten Bilinen ist in diesem Fall aber kein Transfer von Elektronen in den A-Ring möglich und es wird an der C3² Position ein Carbanion gebildet, das protoniert werden muss (Abbildung 23b und 23c). Die Ausbildung einer solchen Bindung wurde bereits für ein künstliches bakterielles Phytochrom mit blauverschobenem Absorptionsmaximum berichtet (Hontani et al. 2016; Shcherbakova et al. 2015). Der hier vorgestellte Mechanismus hat aber in pflanzlichen Phytochromen mehrere Nachteile. So fehlen beispielsweise elektronenziehende Gruppen neben der C3¹ Position des Chromophoren, es werden positive Aminosäuren in der Nähe der C3² Position zur Ausbildung des Carbanion benötigt und dieses Carbanion muss anschließend protoniert werden was auf eine pH-Abhängigkeit der Reaktion hindeutet. Diese Nachteile erklären eventuell, warum BV deutlich schlechter in *AtPhyB*-PSM eingebaut wird als reduzierte Biline und warum ein BV Einbau in *AtPhyB* nicht bereits früher berichtet wurde.

Unabhängig vom tatsächlichen Bindemechanismus stellt sich jedoch die Frage, ob *AtPhyB*-PSM trotz der Verwendung eines anderen Chromophoren immer noch seine biochemische Funktion als lichtgesteuerter Interaktionspartner mit PIF Proteinen ausführen kann. Zu diesem Zweck wurde Größenausschlusschromatographie und Fluoreszenzanisotropie von *AtPhyB*-PSM mit BV bzw. PCB und PIF3-EYFP durchgeführt. Die Messungen nach vorheriger Rotlichtbeleuchtung zeigen, dass Interaktion zwischen den Phytochromen und dem PIF Protein stattfindet (Abbildung 24a und 24b), während diese Interaktion nach Fernrotlichtbeleuchtung stark reduziert ist (Abbildung 24c und 24d). Ein Vergleich der Bindestärken zwischen dem Pfr Zustand und PIF3 weist eine annähernd identische Interaktionsstärke von ca. (250 ± 40) nM für das *AtPhyB* mit BV und (200 ± 70) nM für das *AtPhyB* mit PCB auf (Abbildung 24e und 24f). Die Pr Interaktion ist wie zu erwarten deutlich schwächer mit $> 5 \mu\text{M}$ bzw. $> 2 \mu\text{M}$ für *AtPhyB*-PSM mit BV bzw. PCB.

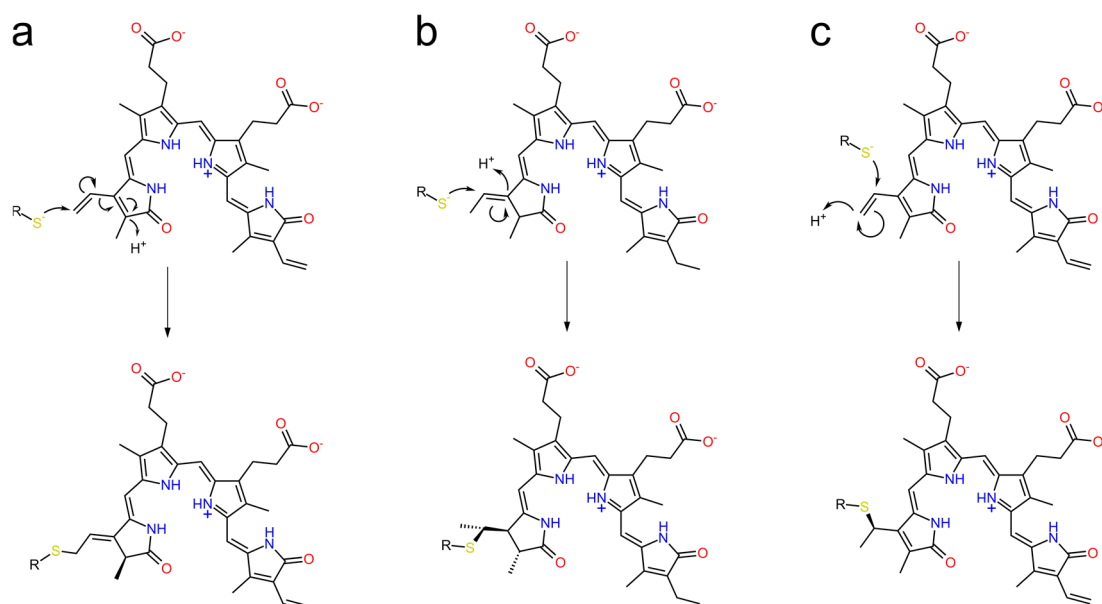


Abbildung 23: Ausbildung der kovalenten Bindungen zwischen verschiedenen Chromophoren und Phytochromen. (a) In bakteriellen Phytochromen erfolgt der nucleophile Angriff des Cysteins der PAS Domäne an der C3² Position von BV. (b) Die kovalente Bindung wird zwischen dem Cystein der GAF Domäne in pflanzlichen Phytochromen und dem C3¹ Atom in reduzierten Bilinen, z. B. PCB, gebildet. (c) Vorgeschlagener Mechanismus für die Ausbildung der kovalenten Bindung zwischen pflanzlichen Phytochromen und BV.

Anhand der mittels *AtPhyB*-PSM mit BV gewonnen Erkenntnisse wurden anschließend eine Phytochromvariante mit einem Histidin an der Position 578 erstellt. Die genannte Position befindet sich innerhalb der Zungenregion und ist im dunkeladaptierten Pr Zustand in räumlicher Nähe zum C3² Atom des Chromophoren (Abbildung 5). Im wildtypischen *AtPhyB*-PSM ist diese Position ein Arginin und trägt somit bereits eine positive Ladung. Ein Histidin in dieser Position könnte jedoch als Protonendonor dienen während gleichzeitig die positive Ladung erhalten bleibt und dadurch den Einbau von BV fördern. Die resultierende BV bindende Phytochrom B Variante (BVB-PhyB) zeigt in der Tat erhöhten BV-Einbau. Unter nicht optimierten Bedingungen war der Einbaugrad von BVB-PhyB um ca. 10 % höher als es dies beim wildtypischen *AtPhyB* der Fall war. Es konnte insgesamt ein Einbaugrad mit BV von 87 % in BVB-PhyB erzielt werden. Außerdem hatte die eingebrachte Mutation keine negativen Auswirkungen auf die biochemische Funktion. BVB-PhyB könnte dadurch von großem Interesse bei der Anwendung des *AtPhyB*-PIF Systems in der Optogenetik und Biotechnologie ohne Notwendigkeit von externer Chromophorzugabe sein.

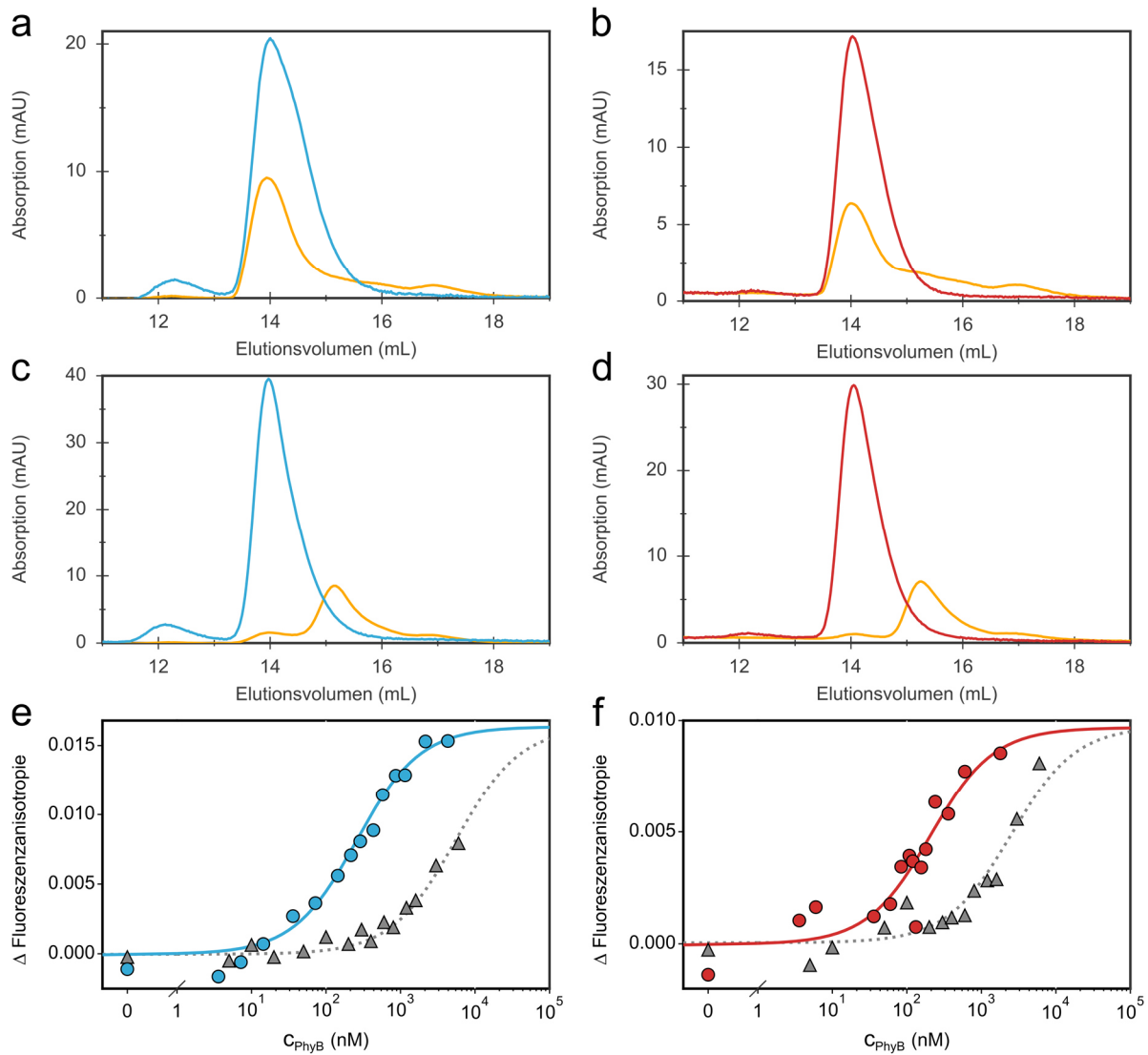


Abbildung 24: Interaktionsstudien an *AtPhyB*-PSM und PIF3-EYFP. (a) Größenausschlusschromatographie von *AtPhyB*-PSM mit BV (blau) und PIF3-EYFP (gelb) Gemischen nach vorheriger Beleuchtung mit Rotlicht. (b) wie in (a) aber *AtPhyB*-PSM mit PCB (rot). (c) Größenausschlusschromatographie von *AtPhyB*-PSM mit BV und PIF3-EYFP Gemischen nach vorheriger Beleuchtung mit Fernrotlicht. (d) wie in (c) aber *AtPhyB*-PSM mit PCB. (e) Fluoreszenzanisotropie von PIF3-EYFP und zunehmenden Konzentrationen von *AtPhyB*-PSM mit BV im Pr (blau) oder Pfr (grau) Zustand. (f) wie in (e) aber *AtPhyB*-PSM mit PCB (rot).

2.4 Identifizierung eines *PhyA* spezifischen Interaktionspartners

A. thaliana besitzt insgesamt fünf Vertreter der Phytochromfamilie, die als *PhyA-E* bezeichnet werden (Bae und Choi 2008). Vor allem für *AtPhyB* wurden viele Partnerproteine entdeckt, die in lichtabhängiger Weise mit dem Phytochrom interagieren (Pham et al. 2018; Khanna et al. 2004). Über mögliche Interaktionspartner der anderen Phytochrome in *A. thaliana* ist weniger bekannt. Es wurde gezeigt, dass die eigentlich *AtPhyB* spezifischen PIF1 und PIF3 zusätzlich ein Erkennungsmotiv für *AtPhyA* besitzen (Zhu et al. 2000; Leivar und Quail 2011). Des

Weiteren wurden noch einige andere *AtPhyA* Partnerproteine entdeckt wie beispielsweise SPA1, die jedoch ebenfalls Bindung zu *AtPhyB* aufweisen (Lu et al. 2015; Sheerin et al. 2015). Für eine orthogonale Anwendung von *AtPhyA* und *AtPhyB* innerhalb der Optogenetik ist daher ein *AtPhyA* spezifischer Interaktionspartner von hohem Interesse. Aus diesem Grund wurden innerhalb eines Kooperationsprojekts (Kapitel 5.4) lichtabhängige Protein-Protein Interaktionsstudien von *AtPhyA* und 30 potenziellen Partnerproteinen mit Hilfe eines Genexpressionssystems mit geteiltem Transkriptionsfaktor durchgeführt. Im Zuge dieser Untersuchung wurde ein Protein mit unbekannter Funktion entdeckt, das nur unter Rotlicht, nicht jedoch im Dunkeln, Interaktion mit *PhyA* aufweist. Da dieses Protein in diesen Untersuchungen keine Interaktion zu *AtPhyB* aufzeigte, wurde es als *only phytochrome A binding* (OPA) Protein benannt.

Beim Versuch OPA lediglich mit dem *AtPhyA*-PSM einzusetzen zeigte sich, dass die Interaktion verloren geht. OPA scheint daher im Gegensatz zur *AtPhyB*-PIF Interaktion nicht mit dem PSM von *AtPhyA* sondern vermutlich dem Effektormodul zu interagieren. Eine Betrachtung der potenziellen Domänenarchitektur von OPA zeigte lediglich eine C-terminale DUF1639 Domäne unbekannter Funktion auf. Diese Domäne ist jedoch nicht an der Interaktion mit *AtPhyA* beteiligt und übernimmt im Organismus daher vermutlich eine andere Funktion. Der für die lichtabhängige Protein-Protein Wechselwirkung mit *AtPhyA* relevante Bereich ist innerhalb der ersten 90 Aminosäuren von OPA kodiert. Die Anwendung des resultierenden verkürzten OPA Konstrukts (sOPA) zusammen mit *AtPhyA* wurde innerhalb des Genexpressionssystems näher charakterisiert und weist in seiner Sensitivität und Reversibilität große Ähnlichkeit zum *AtPhyB*-PIF System auf.

Um die tatsächliche Interaktionsstärken der OPAs mit *AtPhyA* zu bestimmen, wurden *AtPhyA* und OPA-EYFP Konstrukte gereinigt und mittels Fluoreszenzanisotropiemessungen des EYFP untersucht (Abbildung 25). Eine Interaktion zwischen *AtPhyA* und OPAs bewirkt, dass der hydrodynamische Radius der Teilchen zunimmt und daher die Drehbeweglichkeit abnimmt. Dies hat eine Steigerung der Fluoreszenzanisotropie zur Folge, die bei konstanter OPA-EYFP Konzentration und steigender *AtPhyA* Konzentration zur Bestimmung der Bindestärke genutzt werden kann. Für OPA-EYFP ergab sich eine Dissoziationskonstante $K_D = (450 \pm 70)$ nM für den rotlichtadaptierten Pfr Zustand von *AtPhyA*. Der K_D Wert für den Pr Zustand war jedoch > 10 μ M. Diese K_D Werte befinden sich in sehr guter Übereinstimmung mit denjenigen für die *AtPhyB*-PIF Interaktion. Das sOPA hingegen wies reduzierte Affinität von > 10 μ M und > 20 μ M für den Pfr bzw. Pr Zustand auf. Obwohl diese Werte für eine deutlich abgeschwächte

Interaktion sprechen, scheint der Pr/Pfr-Unterschied dennoch groß genug zu sein, um erfolgreich Genexpression lichtabhängig zu steuern.

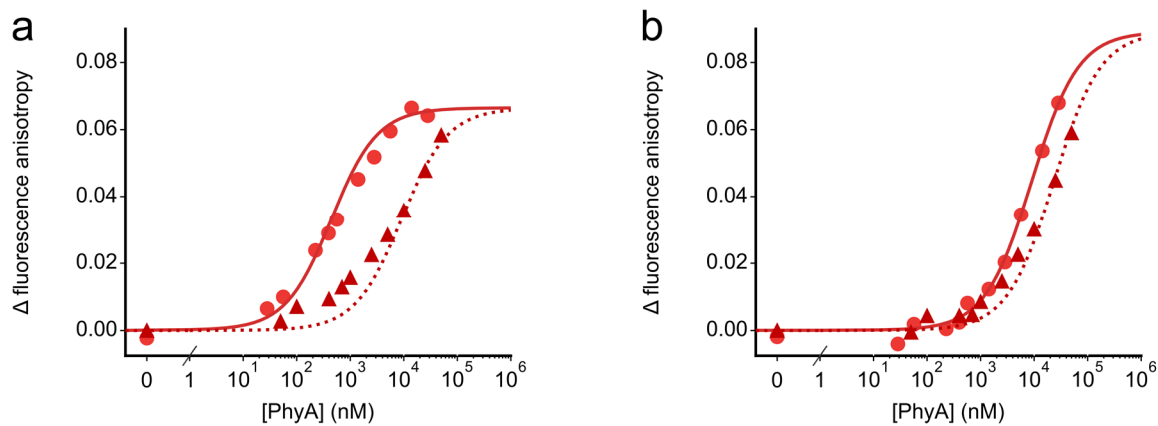


Abbildung 25: Fluoreszenzanisotropie von OPA-EYFP (a) bzw. sOPA-EYFP (b) in Anwesenheit von *AtPhyA* nach vorheriger Beleuchtung mit Rotlicht (Kreise) oder Fernrotlicht (Dreiecke).

Zusammenfassend lässt sich sagen, dass die hier identifizierten OPAs lichtabhängige und selektive Interaktion mit *AtPhyA* eingehen. Das erhaltene *AtPhyA*-OPA System ist damit bis auf die detektierten Wellenlängen orthogonal zum bereits bekannten *AtPhyB*-PIF System. Die identische Lichtspezifität sollte eine kombinierte Anwendung der beiden Systeme erlauben um mehrere Prozesse in Zellen mit nur einer Anregungslichtwellenlänge zu kontrollieren.

2.5 Charakterisierung einer lichtaktivierbaren Phosphatase

Neben der Untersuchung und Weiterentwicklung pflanzlicher Photorezeptoren wurde in einem Kooperationsprojekt auch an der Entschlüsselung der biochemischen Funktion bakterieller Phytochrome gearbeitet (Kapitel 5.5). Bakteriophytochrome sind in der Regel Histidinkinasen, die im Zusammenspiel mit ihren jeweiligen Antwortregulatoren zelluläre Prozesse beeinflussen (Karniol und Vierstra 2003; Bhoo et al. 2001). Obwohl die Histidinkinasefunktion für einige Bakteriophytochrome bereits nachgewiesen wurde, ist die biochemische Funktion des *D. radiodurans* Bakteriophytochroms bis dato unbekannt. *DrBphP* gilt als Modelphytochrome, da innerhalb der letzten Jahre viele grundlegende Erkenntnisse bezüglich der Struktur und des Photozyklus für dieses Phytochrom aufgeklärt wurden (Takala et al. 2014; Ihalaenen et al. 2018). Umso überraschender ist es, dass bis heute nichts über die biochemische Funktion dieses Phytochroms bekannt ist. *DrBphP* besitzt eine Histidinkinasedomäne und der zugehörige Antwortregulator *DrRR* ist wie bei vielen anderen Organismen innerhalb desselben Operons kodiert (Bhoo et al. 2001; Möglich 2019; Arnold et al. 2006). Es wurde daher Interaktionsstudien, enzymatische Tests und Strukturanalysen durchgeführt, um die

tatsächliche Funktion dieses bakteriellen Phytochroms zu entschlüsseln. Zum Vergleich wurde außerdem das Phytochrom AgP1 aus *Agrobacterium fabrum* und dessen Antwortregulator AtRR untersucht, da von diesem Phytochrom bereits bekannt ist dass es als lichtaktivierte Histidinkinase agiert (Karniol und Vierstra 2003).

Zunächst wurde die Interaktion zwischen den Phytochromen und den Antwortregulatoren mittels Größenausschlusschromatographie untersucht (Abbildung 26). Während sich das Elutionsverhalten von *DrBphP* und *DrRR* Gemischen deutlich von dem der Einzelkomponenten unterscheidet, wurde für das AgP1-AtRR Paar kein Unterschied bei der Auftragung als Mischung im Vergleich zu den Analysen der Einzelkomponenten festgestellt. Interessanterweise waren diese Beobachtungen unabhängig vom Beleuchtungszustand der Phytochrome. Um dieses Phänomen besser zu verstehen und um mehr über die tatsächliche Interaktionsstärke der Systeme zu erfahren, wurde anschließend Isotherme Titrationskalorimetrie (ITC) angewandt. Die ITC bestätigte die Beobachtung innerhalb der Größenausschlusschromatographie. Der *DrRR* besitzt eine Interaktionsstärke von $(10.5 \pm 1.5) \mu\text{M}$ mit dem dunkeladaptierten Zustand von *DrBphP*. Im Vergleich dazu wird keine Interaktion zwischen AgP1 und AtRR beobachtet. Zur Ermittlung der Interaktionsstärken im Dunkeln und nach Rotlichtbeleuchtung wurde diese mittels Oberflächenplasmonenresonanzspektroskopie untersucht. Die Interaktion zwischen *DrBphP* und *DrRR* ist lichtunabhängig was durch die annähernd identischen Interaktionsstärken von $(43 \pm 8) \mu\text{M}$ für den dunkeladaptierten Pr Zustand und $(60 \pm 7) \mu\text{M}$ für den lichtadaptierten Pfr Zustand verdeutlicht wird. Im Gegensatz dazu war die AgP1 und AtRR Interaktion unabhängig von den Lichtbedingungen substanziell schwächer und es konnte lediglich abgeschätzt werden, dass diese $> 200 \mu\text{M}$ ist.

Obwohl die Interaktion zwischen AgP1-AtRR System nur transient ist, kann AgP1 seinen Antwortregulator erfolgreich phosphorylieren (Karniol und Vierstra 2003). Der deutliche Unterschied bei den Interaktionsstärken wirft die Frage auf in welcher Art *DrBphP* seine biologische Funktion vermittelt. Ein Test auf Kinaseaktivität mit Hilfe von radioaktiv gelabelten $\gamma\text{-}^{32}\text{P}\text{-ATP}$ verdeutlicht, dass AgP1 erfolgreich seinen Antwortregulator phosphoryliert. Im Gegensatz dazu wird diese Aktivität für *DrBphP* nicht beobachtet. Da Histidinkinasen jedoch gleichzeitig als Kinase und als Phosphatase agieren, ist die Nettoaktivität dieser Enzyme als Summe der jeweiligen Aktivitäten im entsprechenden Signalzustand definiert (Möglich 2019). Sollte *DrBphP* also tatsächlich keine oder nur sehr geringe Kinaseaktivität besitzen, dann wäre es denkbar, dass die Phosphataseaktivität die

treibende Funktion dieses Enzyms ist. Zur Ermittlung der Phosphataseaktivität von AgP1 und DrBphP wurden daher Phos-tag Gele verwendet. Bei diesen Gelen handelt es sich um polymerisierte Acrylamidgele die mit funktionellen Molekülen versetzt wurden. Diese Moleküle sind organische Verbindungen, die Wechselwirkungen mit Phosphatgruppen eingehen und dadurch das Laufverhalten von phosphorylierten Proteinen innerhalb der Natriumdodecylsulfat-Polyacrylamidgelelektrophorese beeinflussen. Nach Behandlung mit Acetyl-Phosphat liegen die Antwortregulatoren phosphoryliert vor, werden anschließend mit den Phytochromen im dunkeladaptierten Zustand oder nach Rotlichtbeleuchtung versetzt, und der Einfluss des Enzyms auf den Phosphorylierungszustand des Antwortregulators wird auf dem Phos-tag Gel untersucht. DrBphP zeigte im dunkeladaptierten Pr Zustand nur leichte Phosphataseaktivität, die durch Rotlichtbeleuchtung aber deutlich gesteigert werden konnte. Für AgP1 wurde für keinen der beiden Zustände eine ausgeprägte Phosphataseaktivität beobachtet (Willett und Kirby 2012).

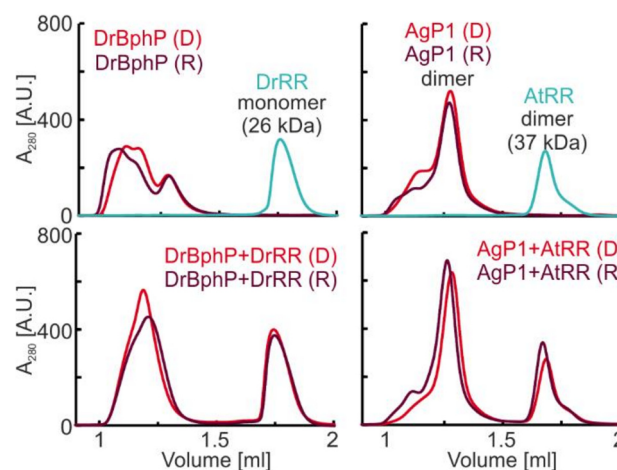


Abbildung 26: Interaktionsstudien zwischen Bakteriophytochromen (BphP) und Antwortregulatoren (RR) aus *D. radiodurans* und *A. fabrum* mittels Größenausschlusschromatographie. Die Komponenten wurden entweder einzeln oder als Gemische aus RR und BphP im dunkeladaptierten Zustand (D) oder nach Rotlichtbeleuchtung (R) untersucht. Die Absorption wurde bei 280 nm detektiert.

Betrachtet man den Zusammenhang zwischen Struktur und biochemischer Funktion so sind die Aminosäuren in näherer Umgebung zum katalytisch aktiven Histidin von hoher Relevanz. Für die Autophosphorylierungsreaktion wird eine Aminosäure mit saurer Ladung in der +1 Position zum aktiven Histidin (H+1) benötigt (Casino et al. 2014). Die Wichtigkeit dieser Position wird durch einen Austausch von Asparaginsäure zu Histidin in AgP1 verdeutlicht, dass dadurch seine Kinaseaktivität verliert. DrBphP besitzt an dieser Stelle ebenfalls ein weiteres Histidin. Das Einbringen einer Asparaginsäure in der H+1 Position von DrBphP konnte die

Kinasefunktion nicht wiederherstellen. Es wird deswegen davon ausgegangen, dass in *DrBphP* noch weitere Aminosäuren verändert werden müssten, um die Kinaseaktivität zu erzeugen. Die Rolle der H+1 Position wird auch verdeutlicht, wenn man die Interaktion mit dem Antwortregulator betrachtet. Befindet sich nämlich an der H+1 Stelle ein Histidin, dann geht die Interaktion mit einem Asparagin innerhalb des Antwortregulators verloren. Eine weitere wichtige Position ist H+4, da innerhalb der HisKA Familie Threonine und Asparagine an dieser Stelle für die Phosphataseaktivität von höchster Wichtigkeit sind (Willett und Kirby 2012). *DrBphP* besitzt an dieser Position eine Glutaminsäure, die bei der Feinregulierung der Phosphataseaktivität hilft, was durch eine Funktionsbeeinträchtigung durch den Austausch mit Alanin bestätigt wurde. Die Glutaminsäure in der H+4 Position interagiert mit dem Mg^{2+} Ion im aktiven Zentrum, eine Position, die in Anwesenheit des phosphorylierten Antwortregulators mit der Phosphatgruppe besetzt ist.

Zusammenfassend lässt sich sagen, dass diese Untersuchung die Frage nach der biochemischen Aktivität von *DrBphP* beantwortet, die seit ca. 20 Jahren ungelöst war (Davis et al. 1999). Des Weiteren eröffnen diese Erkenntnisse die Möglichkeit *DrBphP* als rotlichtaktivierbare Phosphatase innerhalb der Optogenetik zum Einsatz zu bringen.

2.6 Fazit

Die Eigenschaften und Wirkweisen von Phytochromen sind komplex. Obwohl in den letzten Jahren einige Aspekte bezüglich dieser faszinierenden Photorezeptorfamilie aufgeklärt wurden, sind noch viele Gesichtspunkte ungeklärt.

Die hierin präsentierten Erkenntnisse erweitern unser Verständnis von pflanzlichen und bakteriellen Phytochromen. Es werden die Charakteristika von lichtgesteuerten Protein-Protein Interaktionssystemen aufgeklärt, ein neuer Interaktionspartner für *AtPhyA* charakterisiert, ergründet warum pflanzliche Phytochrome Biliverdin vergleichsweise schlecht nutzen und die biochemische Funktion eines bakteriellen Modelphytochroms ergründet.

Die erbrachten Erkenntnisse sind somit von hoher Relevanz für die Grundlagenforschung, da tiefergehende Einsicht in die durch Phytochrome vermittelte Signaltransduktion sowohl für Pflanzen als auch für Bakterien gewonnen wurde. Die Quantifizierung der Interaktionsstärken von Phytochromen und verschiedenen Partnerproteinen wird dazu beitragen das lichtabhängige Zusammenspiel dieser Proteine in Pflanzenzellen besser zu verstehen. Des Weiteren können die hier etablierten Methoden eingesetzt werden, um weitere phytochrombasierte

Wechselwirkungen aufzuklären, um dadurch ein besseres Verständnis dieser Systeme zu erhalten.

Basierend auf diesen Ergebnissen besteht nun die Möglichkeit bereits bestehende optogenetische Systeme zu erweitern oder gänzlich neue lichtschtbare Werkzeuge zu erschließen, was neue Anwendungsmöglichkeiten für die Optogenetik und Biotechnologie eröffnen wird.

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4 Eigenanteil

4.1 Manuskript I

Titel: „Deconstructing and repurposing the light-regulated interplay between *Arabidopsis* phytochromes and interacting factors”

Autoren: Golonka, D.; Fischbach P.; Jena, S. G.; Kleeberg, J.; Essen, L.-O.; Toettcher, J. E.; Zurbriggen, M. D.; Möglich. A.

Veröffentlicht in: Communications Biology (2019), vol. 2, article number: 448, DOI: 10.1038/s42003-019-0687-9

Eigenanteil:

Idee & Konzept: 50 %, Experimente: 60 %, Datenauswertung: 60 %, Abbildungen: 85 %, Verfassen des Manuskripts: 40 %

A. Möglich und D. Golonka hatten die zugrundeliegende Idee. L.-O. Essen beriet bei der Planung. A. Möglich und D. Golonka planten die biochemischen Experimente, M. D. Zurbriggen und P. Fischbach planten Genexpressionsexperimente in Zellkultur, J. E. Toettcher und S. G. Jena planten Membrantranslokationsexperimente in Zellkultur. Plasmide für biochemische Untersuchungen und Anwendung in Genexpressionsexperimenten wurden von D. Golonka und J. Kleeberg erzeugt, S. G. Jena erstellte Plasmide für die Anwendung in Membrantranslokationsexperimenten. Interaktionsstudien in bakteriellem Lysat wurden von D. Golonka und J. Kleeberg durchgeführt. D. Golonka und J. Kleeberg reinigten Proteinvarianten und D. Golonka führte biochemische Analysen durch. P. Fischbach führte Genexpressionsexperimente und S. G. Jena Membrantranslokationsexperimente durch. Die Datenauswertung erfolgte von D. Golonka für biochemische Experimente, P. Fischbach für Genexpressionsexperimente und S. G. Jena für Membrantranslokationsexperimente. D. Golonka und A. Möglich erstellten das Manuskript mit Unterstützung von P. Fischbach, S. G. Jena, L.-O. Essen, J. E. Toettcher und M. D. Zurbriggen. Die korrespondierenden Autoren sind M. D. Zurbriggen und A. Möglich.

4.2 Manuskript II

Titel: „The association kinetics encode the light dependence of *Arabidopsis* phytochrome B interactions”

Autoren: Golonka, D.; Gerken, U.; Köhler, J.; Möglich. A.

Eingereicht bei: Journal of Molecular Biology (in Revision, JMB-D-20-00261)

Eigenanteil:

Idee & Konzept: 25 %, Experimente: 70 %, Datenauswertung: 75 %, Abbildungen: 100 %, Verfassen des Manuskripts: 40 %

A. Möglich, J. Köhler, U. Gerken und D. Golonka hatten die zugrundeliegenden Ideen und planten die Experimente. D. Golonka erstellte Proteine, führte spektroskopische Charakterisierungen und Interaktionsstudien durch. U. Gerken erstellte den Fluoreszenzkorrelationsspektroskopie (FCS) Aufbau und führte mit D. Golonka FCS-Experimente durch. Datenauswertung erfolgte von D. Golonka und U. Gerken. D. Golonka und A. Möglich erstellten das Manuskript mit Unterstützung von U. Gerken und J. Köhler. Die korrespondierenden Autoren sind J. Köhler und A. Möglich.

4.3 Manuskript III

Titel: „*Arabidopsis thaliana* phytochrome B incorporates biliverdin and undergoes light-dependent interactions”

Autoren: Golonka, D.; Kuznetsova, V.; Ihalainen, J. A.; Möglich. A.

Noch nicht eingereicht

Eigenanteil:

Idee & Konzept: 25%, Experimente: 75 %, Datenauswertung: 75 %, Abbildungen: 100 %, Verfassen des Manuskripts: 75 %

A. Möglich und D. Golonka hatten die zugrundeliegende Idee. A. Möglich und D. Golonka planten biochemische Experimente, J. A. Ihalainen und V. Kuznetsova planten Kurzzeit-Spektroskopie-Experimente. D. Golonka erstellte Proteine und führte ihre Charakterisierung und Interaktionsstudien durch. V. Kuznetsova führte Kurzzeit-Spektroskopie-Experimente durch. Datenauswertung erfolgte von D. Golonka und V. Kuznetsova. D. Golonka und V. Kuznetsova erstellten das Manuskript mit Unterstützung von J. A. Ihalainen und A. Möglich. Der korrespondierende Autor ist A. Möglich.

4.4 Manuskript IV

Titel: „Alternative red-light inducible split transcription factor system”

Autoren: Fischbach, P.; Golonka, D.; Fedotov, S.; Hiltbrunner, A.; Möglich. A., Zurbriggen M. D.

Noch nicht eingereicht

Eigenanteil:

Idee & Konzept: 5%, Experimente: 10 %, Datenauswertung: 10 %, Abbildungen: 30 %, Verfassen des Manuskripts: 40 %

M. D. Zurbriggen und A. Hiltbrunner hatten die zugrundeliegenden Ideen. M. D. Zurbriggen und P. Fischbach planten Zellkulturexperimente. A. Möglich und D. Golonka planten Interaktionsstudien an gereinigten Proteinen. P. Fischbach und S. Fedotov führten Zellkulturexperimente, und D. Golonka führte Interaktionsstudien an gereinigten Proteinen durch. Datenauswertung erfolgte durch P. Fischbach für Zellkulturexperimente und durch D. Golonka für Interaktionsstudien an gereinigten Proteinen. P. Fischbach und D. Golonka erstellten das Manuskript und die Abbildungen. A. Möglich und M. D. Zurbriggen editierten das Manuskript. Die korrespondierenden Autoren sind A. Möglich und M. D. Zurbriggen.

4.5 Manuskript V

Titel: „Illuminating a phytochrome paradigm – a light-activated phosphatase in two-component signaling uncovered”

Autoren: Multamäki, E.; Nanekar, R.; Morozov, D.; Lievonen, T.; Golonka, D.; Wahlgren, W. Y.; Stucki-Buchli, B.; Rossi, J.; Hytönen, V. P.; Westenhoff, S.; Ihalainen, J. A.; Möglich, A.; Takala, H.

Eingereicht bei: Cell (noch kein Aktenzeichen)

Eigenanteil:

Idee & Konzept: 5%, Experimente: 5 %, Datenauswertung: 5 %, Verfassen des Manuskripts: 5 %

H. Takala und J. A. Ihalainen hatten die zugrunde liegende Idee. H. Takala, E. Multamäki, A. Möglich und J. A. Ihalainen planten die Experimente. E. Multamäki, R. Nanekar, D. Morozov, T. Lievonen, D. Golonka, W. Y. Wahlgren, B. Stucki-Buchli und H. Takala führten Experimente durch und werteten diese mit Unterstützung von A. Möglich, J. A. Ihalainen, S. Westenhoff, J. Rossi und V. P. Hytönen aus. E. Multamäki, H. Takala, A. Möglich und J. A. Ihalainen erstellten das Manuskript mit Unterstützung der Koautoren. Die korrespondierenden Autoren sind H. Takala und J. Ihalainen.

5 Manuskripte

5.1 Manuskript I

Titel: „Deconstructing and repurposing the light-regulated interplay between *Arabidopsis* phytochromes and interacting factors”

Autoren: Golonka, D.; Fischbach P.; Jena, S. G.; Kleeberg, J.; Essen, L.-O.; Toettcher, J. E.; Zurbriggen, M. D.; Möglich. A.

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ARTICLE

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OPEN

Deconstructing and repurposing the light-regulated interplay between *Arabidopsis* phytochromes and interacting factors

David Golonka¹, Patrick Fischbach², Siddhartha G. Jena³, Julius R.W. Kleeberg¹, Lars-Oliver Essen⁴, Jared E. Toettcher³, Matias D. Zurbriggen^{2*} & Andreas Möglich^{1,5,6,7*}

Phytochrome photoreceptors mediate adaptive responses of plants to red and far-red light. These responses generally entail light-regulated association between phytochromes and other proteins, among them the phytochrome-interacting factors (PIF). The interaction with *Arabidopsis thaliana* phytochrome B (AtPhyB) localizes to the bipartite APB motif of the *A. thaliana* PIFs (AtPIF). To address a dearth of quantitative interaction data, we construct and analyze numerous AtPIF3/6 variants. Red-light-activated binding is predominantly mediated by the APB N-terminus, whereas the C-terminus modulates binding and underlies the differential affinity of AtPIF3 and AtPIF6. We identify AtPIF variants of reduced size, monomeric or homodimeric state, and with AtPhyB affinities between 10 and 700 nM. Optogenetically deployed in mammalian cells, the AtPIF variants drive light-regulated gene expression and membrane recruitment, in certain cases reducing basal activity and enhancing regulatory response. Moreover, our results provide hitherto unavailable quantitative insight into the AtPhyB:AtPIF interaction underpinning vital light-dependent responses in plants.

¹ Lehrstuhl für Biochemie, Universität Bayreuth, 95447 Bayreuth, Germany. ² Institute of Synthetic Biology and CEPLAS, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany. ³ Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA. ⁴ Department of Chemistry, Center for Synthetic Microbiology, Philipps University Marburg, 35032 Marburg, Germany. ⁵ Research Center for Bio-Macromolecules, Universität Bayreuth, 95447 Bayreuth, Germany. ⁶ Bayreuth Center for Biochemistry & Molecular Biology, Universität Bayreuth, 95447 Bayreuth, Germany. ⁷ North-Bavarian NMR Center, Universität Bayreuth, 95447 Bayreuth, Germany. *email: matias.zurbriggen@uni-duesseldorf.de; andreas.moeglich@uni-bayreuth.de

First discovered among the plant photoreceptors¹, phytochromes (Phy) sense red and far-red light to control a range of physiological responses, including seedling germination, shade avoidance, entrainment of the circadian clock, and the transition from vegetative to reproductive growth². Beyond plants, Phys also occur in bacteria and fungi where they mediate chromatic adaptation and pigmentation among other processes^{3,4}. Receptors of the Phy family generally exhibit a bipartite architecture with an N-terminal photosensory core module (PCM) and a C-terminal output module (OPM) (Fig. 1a). The PCM of canonical Phys comprises consecutive PAS (Per/ARNT/Sim), GAF (cGMP-specific phosphodiesterase, adenylyl cyclase, and FhlA), and PHY (Phy-specific) domains and binds within its GAF domain a linear tetrapyrrole (bilin) chromophore^{3,5} (Fig. 1b). Phys of higher plants naturally employ phytochromobilin (PΦB), covalently attached to a cysteine residue within the GAF domain, but can be functionally reconstituted with phycocyanobilin (PCB) of cyanobacterial origin. In darkness, conventional Phys adopt their red-absorbing Pr state with the bilin chromophore in the 15Z configuration; absorption of red light triggers rapid bilin isomerization to the 15E state and population of the metastable, far-red-absorbing Pfr state (Fig. 1b). The Pfr → Pr reversion occurs thermally or can be actively driven by far-red light. Insight from bacterial Phys illustrates that the Z/E isomerization is coupled to refolding of the so-called PHY tongue, a protrusion of the PHY domain, from a β-hairpin to an α-helix conformation, in turn prompting quaternary structural rearrangements^{6–10}. Bacterial Phys mostly form part of two-component signaling cascades¹¹ with OPMs acting as histidine kinases (HKs). By contrast, the Phy OPMs of land plants comprise two PAS domains, PAS-A and PAS-B, and a homologous HK-related domain that, however, lacks key residues essential for function and is thus devoid of HK activity. Rather, plant Phys have been reported to exhibit serine/threonine kinase activity^{12,13}. Plant Phys exert their biological effects via light-regulated cytonucleoplasmic shuttling and protein:protein interactions (PPIs), which manifest in transcriptional responses and proteolytic degradation of cellular target proteins^{14–16}. As one prominent protein family, the so-called phytochrome-interacting factors (PIFs) undergo light-regulated PPIs with plant Phys and act as basic helix–loop–helix transcription factors^{14,17–20} (Fig. 1c and Supplementary Fig. 1).

Arabidopsis thaliana possesses five Phys, denoted AtPhyA–E, that engage with a set of at least eight PIFs, denoted AtPIF1–8^{14,17}. For the arguably best-studied Phy, AtPhyB, preferential interactions of the Pfr state vs. the Pr state were identified with all eight AtPIFs^{14,17,21–23}. Notably, the PCM of AtPhyB is necessary and sufficient for red-light-activated and far-red-light-reversible AtPIF binding^{19,24–26}. Although a pioneering study on the optogenetic use of AtPhyB reported that reversible interactions with AtPIF6 required the presence of PAS-A and PAS-B²⁷, numerous later studies demonstrated that the PCM suffices for photoreversible interactions with AtPIFs^{28–30}. That notwithstanding, the C-terminal OPM likely contributes to light-regulated PPIs and is integral to eliciting physiological responses^{14,15}. Likewise, the light-activated interaction with AtPhyB maps to the weakly conserved APB (active phytochrome B binding) consensus motif within the N-terminal region of AtPIF orthologs that precedes the basic helix–loop–helix domain²⁴ (Fig. 1d). The APB motif consists of two segments, termed APB.A and APB.B, the first of which exhibits higher sequence conservation (Supplementary Fig. 1) and dominates light-activated AtPhyB binding as indicated by site-directed mutagenesis²⁴. In the case of AtPhyA, the isolated PCM also suffices for light-regulated interactions with AtPIF1 and AtPIF3, which localize to the APA motifs (active phytochrome A binding) of these PIFs, somewhat C-terminal of the APB motifs^{31,32}.

Early on, the light-regulated AtPhy:AtPIF PPI has been harnessed for the control of cellular processes in heterologous hosts by red and far-red light^{25,27}, an approach now known as optogenetics³³. As manifold natural processes are intrinsically governed by PPIs, the AtPhy:AtPIF system provides a widely

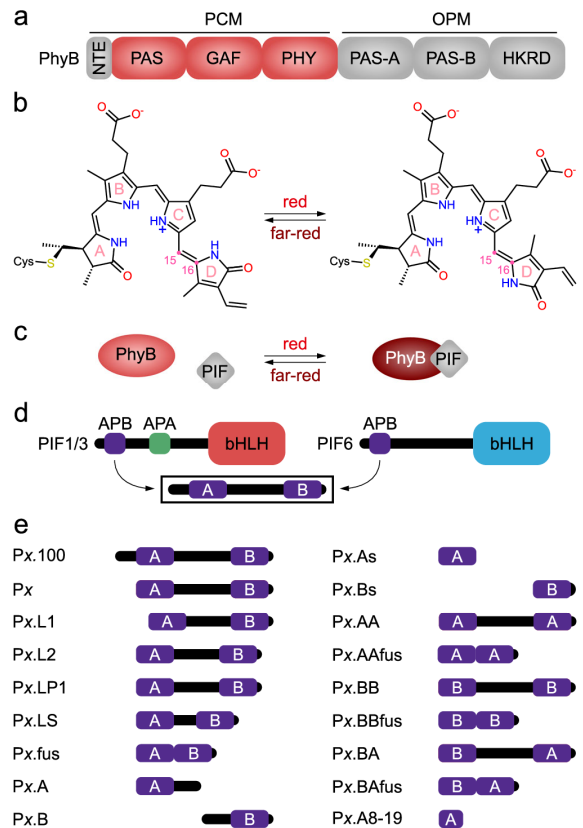


Fig. 1 Architecture and function of plant phytochromes (Phy) and their cognate phytochrome-interacting factors (PIFs). **a** Modular composition of plant phytochromes. An N-terminal extension (NTE) is succeeded by the photosensory core module (PCM) consisting of consecutive PAS, GAF, and PHY domains, with a phytochromobilin (PΦB) chromophore covalently bound as a thioether within the GAF domain. The C-terminal output module (OPM) comprises two additional PAS domains (PAS-A and PAS-B), succeeded by a histidine-kinase-related domain (HKRD). **b** In the dark-adapted Pr (red-absorbing) state of the Phy, the PΦB chromophore adopts its 15Z form. Red light drives isomerization to the 15E form to give rise to the Pfr state (far-red-absorbing). Vice versa, far-red light drives the Pfr → Pr transition. **c** In their Pr state (red), plant Phys show no or at most weak interactions with PIFs. Following red-light absorption, the Pfr state (brown) is populated and affinity for the PIFs enhanced. **d** Modular composition of PIFs. An N-terminal region of around 100 residues contains the so-called APB motif that mediates interactions with phytochrome B. The APB motif further subdivides into the APB.A and APB.B segments²⁴. Certain PIFs also possess a more C-terminal APA motif engaged in interactions with phytochrome A. The C-terminal part comprises a basic helix–loop–helix (bHLH) DNA-binding domain. **e** Based on the N-terminal fragments of *Arabidopsis thaliana* PIFs 3 and 6, a panel of PIF variants were generated and probed for light-dependent protein:protein interactions with the PCM of *A. thaliana* PhyB (cf. Supplementary Table 1 for a detailed description of these derivatives).

applicable means for the bimodal control of cellular phenomena with supreme resolution in space and time³⁴. As a case in point, the expression of transgenes in yeast and mammalian cells has been subjected to red-/far-red-light control via a two-hybrid strategy^{25,35,36}. To this end, a split transcription factor was engineered with one component of the *AtPhyB:AtPIF* pair connected to a sequence-specific DNA-binding domain and the other to a transcriptional *trans*-activating domain. Exposure to red light prompts colocalization of the two entities and onset of expression from synthetic target promoters. In another approach^{27,37,38}, the *AtPhyB:AtPIF* pair conferred light sensitivity on plasma membrane recruitment and cellular signaling cascades in mammalian cells. Although details differ, optogenetic applications to date mostly employ the isolated PCM of *AtPhyB* and the N-terminal 100 amino acids of *AtPIF3/6*, denoted P3.100 and P6.100, that comprise the APB motif.

Despite the eminent role of the *AtPhyB:AtPIF* interaction in nature and optogenetics, quantitative data on the interaction strength and the underlying sequence determinants are scarce. To fill this gap, we dissected and analyzed the light-dependent interaction between *AtPhyB* and *AtPIF3/6* by several qualitative and quantitative approaches. Whereas the *AtPhyB* PCM bound P6.100 with about 10 nM affinity in its Pfr state and showed no detectable affinity in the Pr state, P3.100 exhibited weaker Pfr-state affinity and elevated basal affinity in Pr. By deconstructing *AtPIF3/6* and engineering a wide set of shortened variants, we pinpointed APB.A as decisive for light-regulated PPIs, with a modulatory role for APB.B. Quantitative analyses informed the construction of minimal *AtPIF3/6* fragments of 25 and 23 residues, respectively, that retained stringently light-regulated PPIs with *AtPhyB*. When deployed for the optogenetic control of gene expression and membrane recruitment, the novel *AtPIF* variants with a range of interaction strengths achieved stratified and enhanced light responses.

Results

Deconstructing the *AtPhyB:AtPIF* interaction. Starting from the *AtPIF* constructs P3.100 and P6.100, we generated numerous derivatives with residues deleted from the N terminus, the linker between the APB.A and APB.B segments varied, or either segment omitted or duplicated (Fig. 1e, Supplementary Table 1). All *AtPIF* variants were C-terminally tagged with enhanced yellow fluorescent protein (EYFP) to promote protein solubility and facilitate concentration determination. We implemented a screening assay to efficiently probe interactions of these variants with the Pfr state of the *AtPhyB* PCM. The screen exploits the fact that *AtPIF* binding stabilizes the Pfr state of *AtPhyB* and decelerates the thermal reversion to the Pr state in the dark³⁹ (Fig. 2a). For this assay, the *AtPIF*-EYFP variants were expressed in *Escherichia coli*, purified *AtPhyB* PCM was added to the crude cell lysate in substoichiometric amounts, and the Pfr → Pr reversion kinetics were monitored by absorption spectroscopy (Fig. 2b, c). The initial kinetics were normalized to an EYFP-negative control and provide a convenient readout for interactions (Fig. 2d). Although qualitative in nature, this first screening platform offers important advantages: (i) owing to the specificity of the *AtPhyB:AtPIF* interaction, the assay can be conducted in crude bacterial lysate, without the need for protein purification; and (ii) it can be easily multiplexed to test many variants in a single experiment.

A multiple sequence alignment of the N-terminal regions of *AtPIF1–8* delineates two regions of conservation that define the A and B segments of the APB motif (Supplementary Fig. 1²⁴). The APB.A segment shows stronger conservation and comprises

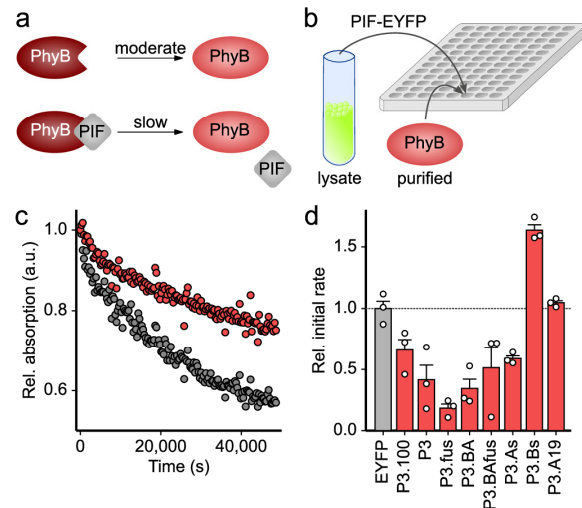


Fig. 2 Screening *AtPIF* variants for protein:protein interactions with the *AtPhyB* PCM. **a** The light-adapted Pfr state (brown) of *AtPhyB* thermally recovers to the dark-adapted Pr state (red) in a moderately paced reaction. When binding to an *AtPIF* variant, the recovery reaction is delayed. **b** *AtPIF* variants were C-terminally tagged with EYFP, expressed in *Escherichia coli*, cells were lysed, and *AtPhyB* PCM was added to the crude lysate. Samples were exposed to red light, and the recovery reaction was monitored over time by absorption measurements. **c** Normalized absorption of the *AtPhyB* PCM measured at 720 nm after red-light absorption in the presence of P3.100 (red) or the EYFP-negative control (gray). **d** The initial rates of the recovery reaction were determined and normalized to the reading obtained for the EYFP-negative control. Data indicate mean $\pm$ SEM of $n = 3$ independent biological replicates.

around 20 residues centered around the consensus core sequence ELXXXXGQ²⁴; by comparison, the APB.B region is considerably shorter and less conserved. As the very N-terminal region preceding APB.A varies substantially among the *AtPIFs* in length and sequence, we deemed it non-essential for *AtPhyB* interactions and removed it from P3.100 and P6.100. The resultant Px variants (here and in the following, $x = 3, 6$) retained interaction with the *AtPhyB* PCM, and all subsequent *AtPIF* variants were thus based on these N-terminally truncated forms (Fig. 2d and Supplementary Fig. 2). Next, we interrogated the linkage between the constituent APB.A and APB.B segments, which is of heterogeneous length and sequence across *AtPIF1–8*. We generated a set of variants, including (i) Px.L1 and Px.L2 in which the linkers of P3/P6 are shortened by 10 residues at their N and C termini, respectively; (ii) Px.LP1 in which said linker is substituted for the corresponding segment of *AtPIF1*, the shortest among all *AtPIFs*; and (iii) Px.LS in which the linker is replaced by a repetitive glycine-serine stretch of 10 residues. As gauged by their effect on dark-reversion kinetics (cf. Supplementary Fig. 2), all these variants still interacted with the Pfr state of the *AtPhyB* PCM. These results imply that the linker connecting the APB.A and APB.B segments is dispensable, which is confirmed in the Px.fus variants that directly link these two segments without any linker and still exhibit interaction with the *AtPhyB* PCM (cf. Fig. 2d and Supplementary Fig. 2). To assess whether productive *AtPhyB* binding mandates a specific topology of the APB segments, we generated the variants Px.BA and Px.BA.fus with

inverted sequential order of APB.A and APB.B, and the original linker sequence kept or removed, respectively. Again, these variants retained interactions with the Pfr state of the *AtPhyB* PCM (cf. Fig. 2d and Supplementary Fig. 2). Site-directed mutagenesis had previously ascribed a dominant role to APB.A in mediating the light-dependent interaction with *AtPhyB*²⁴, and we hence probed the two segments of the composite APB motif separately. Both the APB.A-containing variants Px.A and the Px.As, with or without the N-terminal half of the respective linker, still showed interactions with the *AtPhyB* PCM as judged by the effect on dark-reversion kinetics (cf. Fig. 2d and Supplementary Fig. 2). By contrast, neither the APB.B-based Px.B nor the Px.Bs variants, with or without the C-terminal half of the linker, respectively, exhibited interactions in this assay. Duplication of the A part in the variants Px.AA and Px.AAfus preserved interactions with the *AtPhyB* PCM, and vice versa, duplication of the B segment in Px.BB and Px.BBfus failed to restore them (cf. Supplementary Fig. 2). Taken together, our findings emphasize the dominant role of APB.A for mediating interactions with *AtPhyB*. To further characterize the APB.A segment, we successively trimmed residues flanking its ELXXXXGQ core sequence. However, even the removal of five weakly conserved C-terminal residues in the variants Px.A19 abolished interactions with *AtPhyB*, as judged by their inability to slow down the *AtPhyB*-PCM Pfr → Pr reversion kinetics (cf. Fig. 2d and Supplementary Fig. 2). Likewise, no interaction with the *AtPhyB* PCM was detected for more extensive truncations of the APB.A segment (cf. Supplementary Fig. 2).

Biochemical analyses of the *AtPhyB*:*AtPIF* interaction. The above screening platform affords a qualitative first-pass assessment of the *AtPIF* variants but does not quantify the strength of interactions with *AtPhyB*. Moreover, the assay is limited to interactions within the Pfr state but not the Pr state. We hence selected several of the above *AtPIF* candidates for in-depth analysis. Following expression and purification, we assessed the oligomeric state of these variants and of the *AtPhyB* PCM by size-exclusion chromatography (SEC). In its Pr state, the isolated *AtPhyB* PCM elutes as a monomer with a minor homodimeric fraction, consistent with a recent SEC analysis⁴⁰ (Fig. 3a). In the Pfr state, the predominantly monomeric state is maintained but the retention from the SEC column is slightly delayed, which arguably reflects light-induced conformational changes, i.e., a compaction, of the PCM that may resemble those observed in bacterial Phys^{6–8,10} (Fig. 3b). At a concentration of 10 μ M, P3.100 and P6.100 largely eluted as homodimers with a minor monomeric population (Fig. 3c, Supplementary Fig. 3 and Table 1). Dimerization is not caused by the EYFP tag as the fluorescent protein itself eluted as a monomer (Fig. 3d, Table 1). Notably, the homodimeric state of *AtPIFs* is also observed in nature and critical for their physiological function as basic helix–loop–helix transcription factors⁴¹. Size reduction of the *AtPIFs* impaired homodimerization in several variants to different extent (Supplementary Fig. 3, Table 1). If the APB.A segment was truncated, as in P3.A and P6.A, or excluded altogether, as in P3.Bs and P6.Bs, homodimerization was lost completely. Taken together, these findings point toward a contribution of the APB.A segment to homodimerization of the current *AtPIF* variants and, by extension, of the intact *AtPIF3* and *AtPIF6* proteins⁴¹.

We next investigated the interactions between the *AtPIF3/6* variants and the *AtPhyB* PCM by SEC (Fig. 3e, Supplementary Fig. 4 and Table 1). To this end, we first converted the *AtPhyB* PCM to its Pfr state by illumination with red light (640 nm), incubated it at a 5:1 molar ratio with the different *AtPIF* variants,

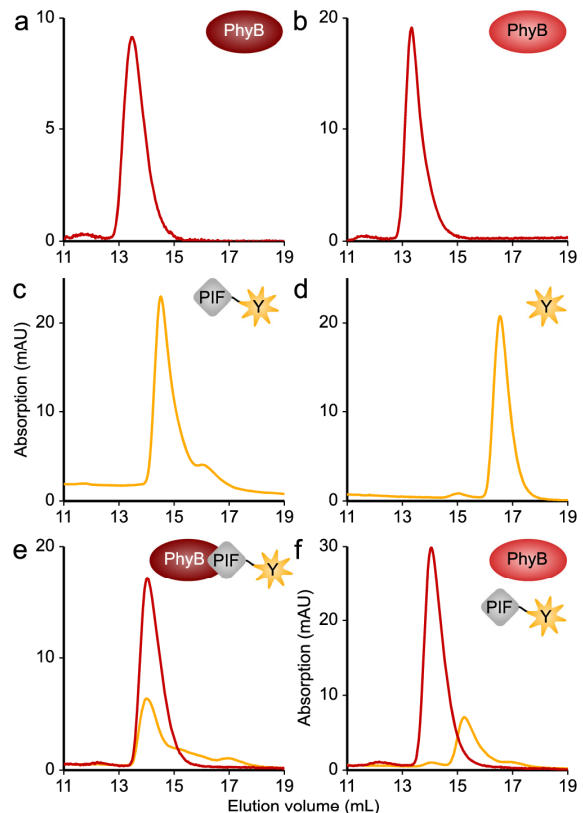


Fig. 3 Oligomeric state of the *AtPIF* variants and light-dependent interactions with the *AtPhyB* PCM. **a** 50 μ M *AtPhyB* PCM were exposed to red light and analyzed by size-exclusion chromatography (SEC), where the yellow and red lines represent absorption at 513 and 650 nm, respectively. **b** As in **a** but the *AtPhyB* PCM was exposed to far-red light prior to chromatography. **c** 10 μ M P3.100-EYFP were analyzed by SEC. Elution profiles were independent of illumination. **d** 10 μ M of the negative control EYFP were analyzed by SEC. Elution profiles were independent of light. **e** A mixture of 10 μ M P3.100-EYFP and 50 μ M *AtPhyB* PCM was exposed to red light and analyzed by SEC. **f** As in **e** but samples were illuminated with far-red light, rather than red light. Experiments were repeated twice with similar results.

and analyzed the mixture by SEC. In full agreement with the first-pass screening (cf. Fig. 2 and Supplementary Fig. 2), all variants that we had identified as binding-competent exhibited interactions with *AtPhyB* PCM at an apparent 1:1 stoichiometry. Vice versa, the *AtPIF* variants that had failed to decelerate *AtPhyB* reversion kinetics (cf. Fig. 2 and Supplementary Fig. 2) lacked any interactions (Supplementary Fig. 4). We also assessed interactions between the *AtPIF* variants and the *AtPhyB* PCM in the Pr state following exposure to far-red light (720 nm) (Fig. 3f and Supplementary Fig. 5). None of the variants showed interactions under these conditions. Insofar red-light-activated binding to the *AtPhyB* PCM had been retained in the truncated *AtPIF* variants, far-red light hence abolished it.

Having engineered a suite of *AtPIF* variants undergoing light-regulated PPIs with the *AtPhyB* PCM, we next sought to quantify the strength of these interaction in both the Pr and Pfr states.

Table 1 Biochemical analyses of the AtPIF3/6 variants.

Name	Oligomeric state ^a	AtPhyB-PCM interaction ^{a,b}	Pfr state K_D (nM) ^c	Pr state K_D (nM) ^c
P3.100	Homodimer	+	200 ± 70	>2000
P6.100	Homodimer	+	10 ± 8	n.d.
P3	Homodimer	+	220 ± 40	>10,000
P6	Homodimer/monomer	+	10 ± 7	n.d.
P3.fus	Monomer	+	270 ± 60	>10,000
P6.fus	Homodimer	+	200 ± 90	>10,000
P3.A	Homodimer	+	220 ± 40	>10,000
P6.A	Monomer	+	280 ± 100	>2000
P3.As	Monomer	+	680 ± 60	>10,000
P6.As	Monomer	+	710 ± 80	>10,000
P3.AA	Homodimer	+	370 ± 40	>3000
P6.AA	Homodimer	+	360 ± 40	>2000
P3.AAfus	Homodimer/monomer	+	230 ± 50	>10,000
P6.AAfus	Homodimer/monomer	+	230 ± 30	>10,000
P3.A19	Monomer	—	>1000	n.d.
P6.A19	Monomer	—	>2000	n.d.
P3.A14	Monomer	—	n.d.	n.d.
P6.A14	Monomer	—	n.d.	n.d.
P3.A8	Monomer	—	n.d.	n.d.
P6.A8	Monomer	—	n.d.	n.d.
P3.B	Monomer	—	n.d.	n.d.
P6.B	Monomer	—	n.d.	n.d.
EYFP	Monomer	—	n.d.	n.d.

n.d. not detectable
^aAs determined by size-exclusion chromatography
^bA “+” sign indicates that an interaction could be detected by size-exclusion chromatography, a “—” sign denotes that no interaction was observed
^cAs determined by fluorescence anisotropy

Notably, detailed quantitative data of that type are largely unavailable but would tremendously improve our understanding of the AtPhyB:AtPIF PPI and inform its optimization. To this end, we resorted to fluorescence anisotropy measurements of the EYFP moiety C-terminally appended to all the AtPIF variants. Binding of a given AtPIF-EYFP variant to the AtPhyB PCM would increase its effective hydrodynamic radius, slow down rotational diffusion, and thus increase fluorescence anisotropy (Fig. 4a). We hence incubated a constant 20 nM of the AtPIF-EYFP variants with increasing amounts of AtPhyB PCM under red or far-red light and recorded binding isotherms. The reference construct P6.100 exhibited strong binding to the AtPhyB PCM under red light but no detectable binding under far-red light even at AtPhyB-PCM concentrations of 2 μM (Fig. 4b). When calculating dissociation constants (K_D), one must consider that red light not only drives the Pr → Pfr transition of Phys but also the reverse Pfr → Pr process. Consequently, continuous illumination with red light (640 nm) leads to population of a photostationary state with a mixed Pfr/Pr population at a ratio of ~0.56/0.44⁴² (Fig. 4c). Correcting for the actual fraction in the Pfr state, we determined a K_D for the P6.100:AtPhyB-PCM pair of 10 ± 8 nM (Table 1). This value is in good agreement with an earlier estimate for this pair of 20–100 nM within mammalian cells based on fluorescence microscopy²⁷. In comparison to P6.100, P3.100 exhibited a weaker K_D of 200 ± 70 nM in Pfr and an elevated basal affinity in Pr, with an estimated K_D on the order of low micromolar (Fig. 4d and Table 1). This residual interaction could in principle be due to partial population of the Pfr state of the AtPhyB PCM under the chosen illumination conditions; however, the absence of basal affinity in case of P6.100 strongly argues against this notion. The slightly weaker affinity and much less pronounced light effect in P3.100 compared to P6.100 may account for the previously reported inability to detect light-regulated interactions of AtPIF3 with the AtPhyB PCM in mammalian cells²⁷. We then recorded

binding isotherms under red and far-red light for all the AtPIF variants we had purified and analyzed by SEC (Supplementary Figs. 4 and 5, Table 1). Consistent with our first-pass assessment (cf. Fig. 2d and Supplementary Fig. 2), the removal of the non-conserved N-terminal residues preceding the APB.A segment had no influence on the Pfr interaction. Unexpectedly, omission of these residues in the AtPIF3 context substantially attenuated the basal Pr-state affinity. For the AtPIF3 variants, removal of the linker and the APB.B part had no or at most modest effects on affinity to the Pfr state (Supplementary Fig. 6, Table 1). By contrast, in AtPIF6, the removal of the linker and the APB.B part more severely attenuated the affinity to the Pfr state to values between 200 and 700 nM. In addition, the affinity to the Pr state, non-detectable for the variants P6.100 and P6, increased as well. As a corollary, AtPIF3 and AtPIF6 variants lacking the APB.B segment exhibited closely similar K_D values for a given construct topology. As a case in point, the P3.As and the P6.As variants, comprising 25 and 23 residues, respectively, both interacted with the AtPhyB PCM with an affinity of ~700 nM in the Pfr and weaker than 10 μM in the Pr state. These data for P6.As are consistent with a recent report that demonstrated light-dependent PPI for an AtPIF6 construct of closely similar length and sequence⁴³. Duplication of the APB.A segments in the AtPIF3/6 backgrounds resulted in variants with affinities in the range of 200–400 nM for Pfr and weaker than 2 μM for Pr. We also analyzed several AtPIF3/6 variants entirely lacking the APB.A segment or possessing shortened versions of it, neither of which showed any interaction with AtPhyB PCM when probed by SEC nor by their effect on Pr reversion kinetics. In almost all these variants, fluorescence anisotropy failed to detect interactions either (Supplementary Fig. 6 and Table 1); merely, the P3.A19 and P6.A19 variants with C-terminally trimmed APB.A segments exhibited weak affinity for the Pfr state in the low micromolar range (Supplementary Fig. 6 and Table 1). In summary, these results

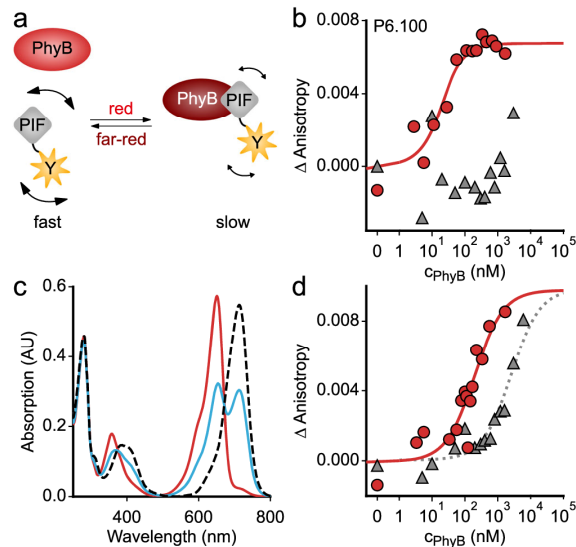


Fig. 4 Quantitative analyses of the light-dependent protein:protein interaction between *AtPIF* variants and the *AtPhyB* PCM. **a** In its Pr state, the *AtPhyB* PCM exhibits weak or no affinity to *AtPIF*, but upon red-light exposure, the affinity is enhanced. Binding to the *AtPhyB* PCM increases the effective hydrodynamic radius of the *AtPIF* variants and slows down rotational diffusion. In turn, the fluorescence anisotropy of an EYFP tag C-terminally appended to the *AtPIF* increases. **b** Titration of 20 nM P6.100-EYFP with increasing concentrations of dark-adapted (gray) or red-light-exposed *AtPhyB* PCM (red), as monitored by anisotropy of the EYFP fluorescence. Data points show mean of $n = 3$ biological replicates. The red line denotes a fit to a single-site-binding isotherm. **c** Absorption spectra of the *AtPhyB* PCM in its dark-adapted Pr state (red line) and as a Pfr/Pr mixture following red-light exposure (blue). The dashed line denotes the absorption spectrum of the pure Pfr state, calculated according to ref. ⁴². **d** As in **b** but for P3.100-EYFP rather than P6.100-EYFP. Experiments were repeated twice with similar results.

confirm the APB.A segment as the main interaction epitope in both *AtPIF3* and *AtPIF6*. Intriguingly, *AtPIF6* differs from *AtPIF3* by higher affinity for Pfr and much reduced affinity for Pr. As the removal of the APB.B segment largely cancels these differences, we conclude that APB.B in *AtPIF6*, but not in *AtPIF3*, enhances the affinity for Pfr and diminishes that for Pr. In *AtPIF3*, the N-terminal amino acids contribute to elevated basal affinity for Pr.

Repurposing the *AtPhyB:AtPIF* interaction for optogenetics. Through sequence variations and quantitative analyses, we generated modules for light-regulated PPIs spanning an affinity for the Pfr state from around 10 to 700 nM. We next investigated whether this set of novel *AtPIF* variants can be leveraged for optogenetics in mammalian cells. In a first line of experiments, we embedded the variants into a previously reported system for red-/far-red-light-regulated gene expression that provides an in-cell readout of relative PPI affinities^{36,44}. To this end, the *AtPhyB* PCM was covalently attached to a VP16 *trans*-activating domain, and the different *AtPIF* variants were linked to the E-protein DNA-binding domain, which binds to a cognate operator sequence upstream of a minimal promoter driving expression of secreted alkaline phosphatase (SEAP) (Fig. 5a). Through

light-induced *AtPhyB:AtPIF* interactions, the *trans*-activating domain localizes to the DNA-binding domain and the promoter and thereby induces SEAP expression. SEAP activity levels are quantified and normalized to the levels of constitutively expressed *Gussia* luciferase to correct for variations of cell density, transfection efficiency, and overall expression. We found that the P3.100 and P6.100 reference constructs upregulated normalized SEAP expression by tenfold and fourfold, respectively, under red light compared to darkness when expressed in Chinese hamster ovary cells (CHO-K1). The comparatively small regulatory effect for P6.100 results from substantial basal SEAP expression. We then subjected all the *AtPIF3/6* variants we had previously characterized to the same analysis (Fig. 5b, c and Supplementary Fig. 7). Consistent with the above measurements, *AtPIF* variants that lacked detectable interactions with the *AtPhyB* PCM, e.g., Px.B and Px.A19, failed to stimulate reporter expression regardless of illumination. By contrast, variants that exhibited interactions with the *AtPhyB* PCM were generally capable of inducing SEAP expression under red light, albeit to different degree. Overall, the expression levels observed for the individual *AtPIF* variants scaled with binding affinity, in that low measured K_D values correlated with strong SEAP activity. For instance, all *AtPIF3/6* variants containing the intact APB.A segment exhibited strong expression under red light. Whereas P6.100 suffered from relatively high basal expression, the shortened *AtPIF6* derivatives generally showed reduced SEAP expression in darkness, translating into much more pronounced regulatory effects. For instance, in the variant P6.A the SEAP expression increased by 43-fold under red light relative to darkness. Duplication of APB.A in the variants P6.AA and P3.AA elevated SEAP expression under red light and, to lesser extent, in darkness, thereby enhancing the regulatory effect. The overall higher SEAP expression under red light for these variants could reflect the binding of two *AtPhyB*-VP16 modules to one Px.AA protein. However, we note that, under the conditions employed for the SEC analysis, we did not find evidence for simultaneous binding of two *AtPhyB* PCM entities to the Px.AA variants. We also assessed the photoreversibility of the gene-expression systems based on the *AtPIF* derivatives (Supplementary Fig. 8). When the cells were first exposed to red light for 24 h, followed by far-red illumination for another 24 h, they exhibited basal SEAP expression levels comparable to cells incubated in darkness throughout. Given that gene expression for the different sequence variations followed similar trends in both the *AtPIF3* and the *AtPIF6* backgrounds, we wondered whether the emerging underlying principles extend to other *AtPIF* orthologs. We hence generated the corresponding sequence variations in the *AtPIF1* background and assessed their impact on light-regulated gene expression (Fig. 5d and Supplementary Fig. 7). Several of the resultant *AtPIF1* variants supported light-activated SEAP expression, although generally with slightly attenuated maximal levels and regulatory effects. Nonetheless, the *AtPIF1* variants conformed to the general activity pattern observed for the *AtPIF3/6* variants; specifically, only the *AtPIF1* variants preserving an intact APB.A segment were capable of upregulating SEAP expression under red light. Taken together, these experiments demonstrate the utility of the cellular set-up for the efficient appraisal of light-regulated PPIs in mammalian cells. By capitalizing on this set-up, we obtained derivative systems with enhanced dynamic range and reduced leakiness that outperformed the original reference systems.

In a second set of experiments, we deployed several of the newly generated *AtPIF6* variants for light-regulated recruitment of target proteins to the plasma membrane of NIH-3T3 cells. To this end, we equipped the *AtPhyB* PCM with a C-terminal CAAX prenylation motif for membrane targeting and the *AtPIF6*

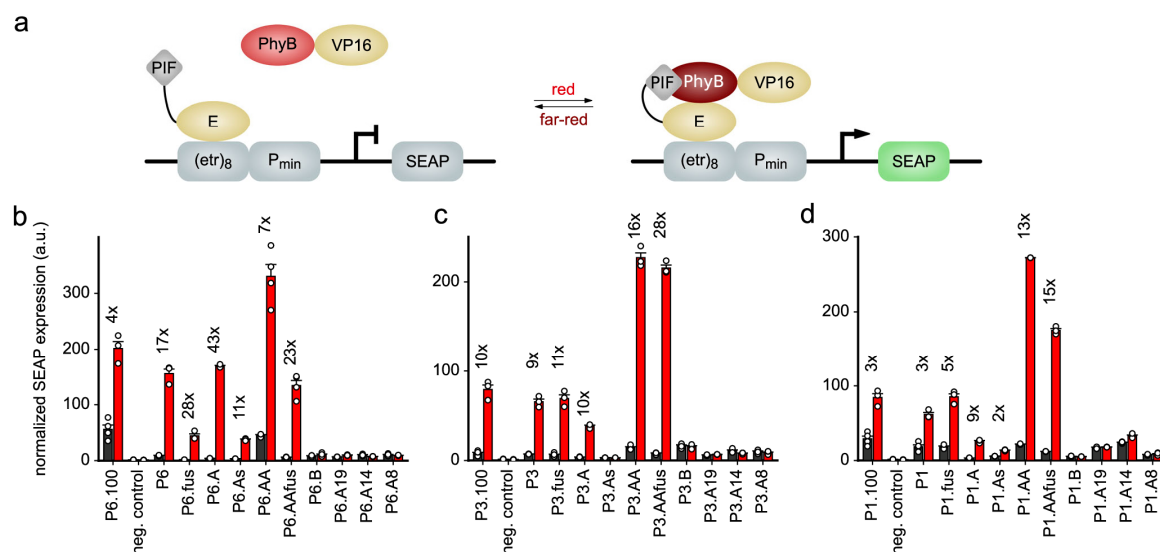


Fig. 5 Harnessing the AtPIF variants for the light-dependent regulation of gene expression in mammalian cells. **a** The AtPhyB PCM and AtPIF variants are connected to a VP16 *trans*-activating domain and an E-protein DNA-binding domain that binds to a synthetic promoter sequence. Red light promotes association of the AtPhyB:AtPIF pair and thereby activates the expression of a secreted alkaline phosphatase (SEAP) reporter gene. **b** SEAP expression was determined in Chinese hamster ovary cells (CHO-K1) for the diverse AtPIF6 variants and normalized to the constitutive expression of *Gaussia* luciferase. Black and red bars denote mean $\pm$ SEM normalized SEAP expression for $n = 4$ independent biological replicates under dark or red-light conditions, respectively. Cells were kept in darkness for 24 h, supplemented with PCB, and then either kept in darkness for 24 h or illuminated for 24 h with $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ 660-nm light. As a negative control, the reporter construct alone was transfected. The numbers above the bars indicate the factor difference between dark and red-light conditions for a given AtPIF6 variant. **c** As **b** but for the AtPIF3 variants. **d** As **b** but for the AtPIF1 variants.

variants with an N-terminal EYFP tag^{27,37,38} (Fig. 6a). Cell lines stably expressing both the AtPhyB PCM and one of the AtPIF6 variants, linked by an internal ribosome entry site (IRES), were created through lentiviral transduction. Cells were exposed to red (650 nm) and far-red light (750 nm), respectively, and the subcellular distribution of the EYFP-AtPIF6 variants was monitored by fluorescence microscopy (Fig. 6b–e). Under far-red light, the reference variant P6.100 mostly localized to the cytoplasm, but under red light it partially translocated to the plasma membrane (Fig. 6c–f). Whereas the variants P6.A, P6.As, and P6.AA exhibited overall similar subcellular distribution under red and far-red light as P6.100, the variant P6.fus failed to show any light response of subcellular localization. Although subtle performance differences between the individual AtPIF6 variants cannot be ruled out, these are exceeded by the cell-to-cell variability of light-dependent translocation (Fig. 6f). Nonetheless, the experiments show that the new AtPIF6 variants with a much smaller footprint support light-regulated plasma membrane recruitment at similar efficiencies as the reference P6.100. This notion is further supported by the overall comparable expression level of the AtPIF6 variants and its effect on the magnitude of light-regulated membrane recruitment (Fig. 6g).

Discussion

In this study, we have dissected the light-regulated PPIs between the AtPhyB PCM and the AtPIFs 3 and 6, which underpin diverse adaptive responses in planta and multiple applications in optogenetics. To this end, we implemented a set of complementary experimental approaches ranging from SEC and fluorescence anisotropy to reporter assays in mammalian cells that deliver

both qualitative and quantitative information on the PPIs. At a qualitative level, these assays consistently showed the APB.A segment to be necessary and sufficient for AtPhyB-PCM interactions, in line with previous reports²⁴. By contrast, the APB.B segment alone did not promote detectable interactions. Our quantitative analyses put concrete numbers on the affinity of the AtPhyB:AtPIF3/6 pairs, information that hitherto was largely lacking. Strikingly, P6.100 exhibited a K_D of only ~ 10 nM for AtPhyB PCM in its Pfr state but entirely lacked interaction with the Pr state, from which we estimate an at least 1000-fold affinity difference. By contrast, the light dependence of the P3.100: AtPhyB-PCM interaction was less pronounced, with dissociation constants of ~ 200 nM in the Pfr state and low micromolar in the Pr state. We tied the more stringent red-light response in AtPIF6 to its APB.B segment, which enhances affinity for the Pfr state of the AtPhyB PCM while simultaneously attenuating basal affinity for the Pr state. We speculate that these inherent differences between AtPIF3 and AtPIF6 might reflect their natural roles in planta. Whereas AtPIF3 predominantly serves as a negative regulator of photomorphogenesis by modulating the abundance of AtPhyB^{45–47}, AtPIF6 acts as a positive regulator by inhibiting hypocotyl elongation under red light, at least when over-expressed⁴⁸. To prevent untimely inhibition of hypocotyl growth, a more stringent light response with very low basal affinity in Pr may be required for this particular PIF. Recently, it has been reported that PIFs, and in particular AtPIF3, are constantly turned over both in darkness and under red light as a mechanism of achieving optimal levels for tight regulation of the skotomorphogenic and photomorphogenic responses¹⁴. A more permissive binding of AtPIF3 to the Pr state of AtPhyB as observed here

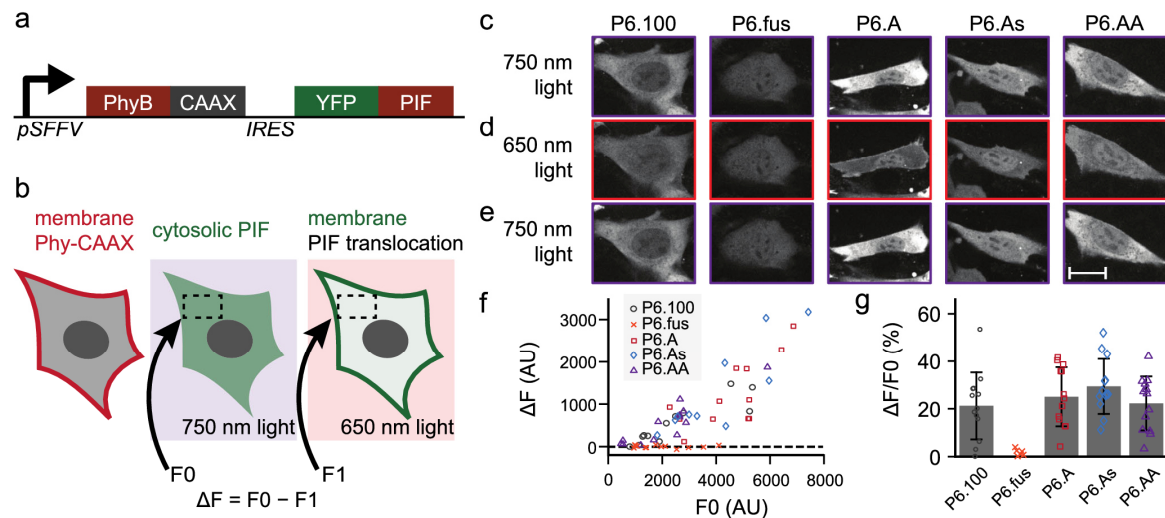


Fig. 6 Photoreversible recruitment to the plasma membrane. **a** AtPhyB and one of the several new AtPIF6 variants, equipped with a C-terminal CAAX prenylation motif or an N-terminal EYFP tag, respectively, were encoded on a bicistronic vector with an intervening IRES sequence and expressed in NIH-3T3 cells. **b** Owing to the CAAX tag, AtPhyB localizes to the plasma membrane, while the EYFP-AtPIF6 variants shuttle between cytosol and plasma membrane as a function of light. Under far-red light (750 nm), the EYFP-AtPIF6 variants exhibit cytosolic localization; under red light (650 nm), they can bind to AtPhyB and translocate to the membrane. **c–e** Fluorescence micrographs of NIH-3T3 cells expressing AtPhyB-CAAX and different EYFP-AtPIF6 variants under far-red light (**c**), after red-light exposure (**d**), and after additional exposure to far-red light (**e**). The scale bar denotes 20 μ m. **f** The relative depletion of cytosolic EYFP fluorescence under red light compared to far-red light for the EYFP-AtPIF6 variants. Data represent mean $\pm$ SD of $n \geq 12$ individual cells. **g** Dependence of the relative fluorescence change on the overall EYFP-AtPIF6 expression level.

might facilitate the regulation of PIF abundance in darkness. This concurs with reports that AtPhyB mediates phosphorylation by PPK-family kinases and subsequent degradation of AtPIF3 in both the Pr and Pfr states⁴⁹. The differential affinities of the individual PIFs might therefore contribute to the fine-tuning of physiological responses^{14,49}. In fact, our study now provides a means of gradually adjusting the interaction strength of a given PIF, which could benefit the analysis of signal transduction mechanisms in planta. In a similar vein, the quantitative data on the AtPhyB:AtPIF PPI may help rationalize the phenotypes of pertinent *pif* mutant alleles. Finally, the comparatively smaller regulatory effect in AtPhyB:AtPIF3, compared to AtPhyB:AtPIF6, may explain why this PPI pair proved inferior for generic optogenetic applications²⁷.

By deconstructing and quantitatively analyzing AtPIF3/6, we devised a suite of interaction modules with several beneficial traits (Table 1): First, the AtPIF variants span an affinity range from 10 to 700 nM, thus enabling the precise tuning of light-regulated PPIs as demanded by a specific application. Second, the AtPIFs can be reduced to around 23–25 residues while largely retaining light-regulated PPIs with the AtPhyB PCM. As we demonstrate, the smaller size facilitates the construction of tandem repeats of the APB.A motif, which, depending upon context, may enhance light-dependent responses. Third, the reduction in size also affected the oligomeric state of the AtPIFs, which are homodimeric at full length⁴¹ but predominantly monomeric in several of the truncated variants studied presently. As we showcase for the scenarios of light-regulated gene expression and membrane recruitment, the set of novel AtPIF variants can indeed improve absolute activity and degree of light regulation in optogenetics. As a case in point, despite stringently light-regulated PPIs with the AtPhyB PCM, the original P6.100 variant promoted substantial basal gene expression in darkness, thus degrading the regulatory effect of light. We tentatively ascribe the relatively poor performance of P6.100 to its

high Pfr-state affinity; even limited population of the AtPhyB Pfr state, e.g., due to light pollution or temperature changes⁵⁰, may hence activate the PPI to considerable extent and over prolonged periods³⁹. In support of this notion, the attenuation of the Pfr-state affinity in the shortened AtPIF6 variants led to reduced basal activity and enhanced regulatory efficiency. Duplication of the APB.A segment improved the performance for light-regulated expression, although the Pfr-state affinity of the Px.AA variants is almost unchanged relative to the corresponding Px.A variants. We hence ascribe this improvement to avidity and cooperativity effects. Our analyses readily extended to the AtPIF1 context, where shortened variants exhibited similar patterns of activity and light regulation as the AtPIF3/6 variants (cf. Fig. 5b–d). We speculate that the underlying principles can be generalized to APB-containing PIF proteins from *A. thaliana* and other plants^{51,52}. The performance of individual AtPIF variants in a given experiment can considerably vary and may be difficult to gauge upfront, not least because it likely depends on application context. We thus consider it an advantage to have now a set of AtPIF variants with known interaction strengths and varying properties. With this suite of AtPIF variants in hand, additional processes may be unlocked for optogenetic control by red and far-red light. As recently summarized⁵³, numerous cellular parameters and pathways depend on PPIs and can thus be controlled by certain photoreceptors that associate or dissociate under blue light. The underlying regulatory strategy should readily extend to the present AtPhyB:AtPIF pairs and thereby to red and far-red light. Other potential use cases for the new AtPIF variants include immunoreceptor signaling³⁰ and light-regulated biomaterials⁵⁴. As one shortcoming, optogenetic applications of plant Phys currently require the exogenous addition of PCB or PΦB chromophores, which do not widely occur outside cyanobacteria and plants. This contrasts with bacterial Phys, which utilize biliverdin (BV) that is available in mammals as a heme degradation

product^{55–58}. In particular, a recently described bacterial Phy undergoes PPIs depending on red and far-red light and has been harnessed for light-regulated gene expression^{59–61}. The reliance on BV in this system obviates exogenous chromophore addition, which may prove advantageous for applications in vivo.

In summary, we have constructed and characterized a toolkit of novel AtPIF variants with varying interaction strength, size, and oligomeric state. Beyond application in optogenetics, the availability of these variants also stands to benefit the biophysical analyses of the Phy:PIF interaction. Although previous studies had localized this interaction to the N-terminal extension of Phys, atomically resolved information on the Phy:PIF complex is lacking^{40,62–64}. Minimized AtPIFs may well facilitate X-ray crystallographic analysis and thus pave the way toward elucidation of the complex structure. Moreover, the qualitative and quantitative interaction assays presently established can be deployed to chart Phys and interacting factors from *A. thaliana* and other plants.

Methods

Molecular biology and protein purification. Genes encoding *A. thaliana* PhyB PCM (residues 1–651), PIF3 (1–100), and PIF6 (1–100) were synthesized with codon usage adapted for expression in *E. coli* (GeneArt, Invitrogen, Regensburg, Germany). Via Gibson assembly⁶⁵, the AtPhyB PCM was furnished with a C-terminal hexahistidine tag and subcloned onto the pCDFDuet1 vector (Novagen, Merck, Darmstadt, Germany) under control of a T7-lacO promoter; the plasmid, designated pDG282, additionally harbors a bicistronic cassette of *Synechocystis* sp. heme oxygenase 1 and pcyA⁶⁶, also under the control of T7-lacO. For the expression of AtPIF3/6, the corresponding genes were subcloned onto a pET-19b vector (Novagen) under the control of a T7-lacO promoter by Gibson assembly or AQUA cloning⁶⁷ and thereby equipped with an N-terminal His₆-SUMO tag⁶⁸ and a C-terminal EYFP tag, attached via a short linker (DSAGSAGSAG). For interaction studies in bacterial lysate, the AtPIF3/6 genes were subcloned onto a pET-28c vector (Novagen) under the control of a T7-lacO promoter, again with C-terminal linkers and EYFP. Variants of the AtPIF proteins were generated in both plasmid contexts, and the identity of all constructs was confirmed by Sanger DNA sequencing (GATC, Konstanz, Germany or Microsynth Seqlab, Göttingen, Germany).

For AtPhyB expression, the plasmid pDG282 was transformed into the *E. coli* BL21(DE3) strain. Transformant cells were grown in 2 × 1000 mL terrific-broth (TB) medium, supplemented with 100 µg mL^{−1} streptomycin, at 37 °C in darkness until an optical density at 600 nm (OD₆₀₀) of 0.6–0.8 was reached. δ-Aminolevulinic acid was added at 0.5 mM to assist chromophore production⁶⁹, and the expression was induced by adding 1 mM β-D-1-thiogalactopyranoside (IPTG). Cultivation continued overnight at 18 °C, before cells were harvested by centrifugation, resuspended in lysis buffer [50 mM Tris/HCl pH 8.0, 20 mM NaCl, 20 mM imidazole; supplemented with protease inhibitor mix (cOmplete Ultra, Roche Diagnostics, Mannheim, Germany)], and lysed by sonification. The cleared lysate was purified by immobilized ion affinity chromatography (IMAC) on Protino Ni-NTA 1 mL columns (Macherey-Nagel, Düren, Germany) and eluted with a linear imidazole gradient from 20 to 500 mM. Elution fractions were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), where 1 mM Zn²⁺ was added to enable detection of covalently incorporated bilin chromophores via zinc-induced fluorescence⁷⁰. Suitable fractions were pooled and dialyzed overnight into AEX buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl, 5 mM 2-mercaptoethanol), applied to a HiTrap Q HP 1 mL anion-exchange column (GE Healthcare Europe GmbH, Freiburg, Germany), and eluted using two successive linear gradients from 50 to 300 mM NaCl and from 300 to 500 mM. Eluted fractions were analyzed by PAGE, appropriately pooled, dialyzed against storage buffer [10 mM Tris/HCl pH 8, 10 mM NaCl, 10 % (w/v) glycerol], and stored at −80 °C.

Purification of the AtPIF3/6-EYFP variants employed a similar protocol with the following differences. No δ-aminolevulinic acid was added, and incubation after induction continued at 16 °C for 40 h. Following IMAC, the N-terminal His₆-SUMO was cleaved overnight at 4 °C during dialysis into 50 mM Tris/HCl pH 8.0 and 20 mM NaCl using SENP2-protease. The His₆-SUMO tag was removed by IMAC, and the flow-through containing the AtPIF3/6 construct was collected and analyzed by PAGE. Depending upon purity, the proteins were optionally further purified by anion-exchange chromatography as described above. Pure AtPIF3/6-EYFP variants were dialyzed into storage buffer and stored at −80 °C. An analysis by denaturing PAGE of the purified AtPIF3/6-EYFP constructs and the AtPhyB PCM is shown as Supplementary Fig. 9.

Spectroscopic analysis. The concentration of purified AtPhyB PCM and the AtPIF3/6-EYFP variants were determined at 22 °C by absorption measurements on an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany). In case of the AtPIF3/6-EYFP variants, a molar extinction coefficient at 513 nm of 84,300 M^{−1} cm^{−1} was used⁷¹. Photoreversible

Pr ↔ Pfr conversion of AtPhyB PCM was ascertained by illumination with light-emitting diodes (LED) with emission wavelengths of 650 ± 15 nm (5.6 µW cm^{−2}) and 720 ± 15 nm (0.7 µW cm^{−2}), respectively. Spectra recorded after illumination revealed isosbestic points at 374 and 672 nm. Absorption spectra were also recorded after denaturation in 6.5 M guanidinium hydrochloride. By referencing to the previously reported extinction coefficient for PCB under these conditions⁷², we calculated an extinction coefficient at the isosbestic point 672 nm for AtPhyB PCM in its native state of 47,600 M^{−1} cm^{−1}. The fraction of AtPhyB PCM in the Pfr state upon saturating red-light illumination (640 nm) was determined as described in ref. 42.

Interaction assay in bacterial lysate. pET-28c plasmids harboring AtPIF3-EYFP or AtPIF6-EYFP variants were transformed into chemically competent BL21(DE3) cells. Three replicate clones were used to inoculate 3 × 5 mL TB medium supplemented with 50 µg mL^{−1} kanamycin. Cultures were incubated at 37 °C up to an OD₆₀₀ of 0.6–0.8, at which point temperature was lowered to 16 °C and expression was induced by addition of 1 mM IPTG. Incubation continued overnight, and cells were harvested by centrifugation at 3000 × g for 10 min. Pelleted cells were resuspended in 300 µL lysis buffer [1× FastBreak Cell Lysis Reagent (Promega GmbH, Mannheim, Germany), 10 µg mL^{−1} DNaseI (PanReac AppliChem, Darmstadt, Germany), 200 µg mL^{−1} lysozyme (Sigma-Aldrich, Darmstadt, Germany)] and rotated at 22 °C for 10 min. Cell debris was removed by centrifugation at 186,000 × g for 45 min using an Optima MAX-XP Ultracentrifuge (Beckman-Coulter, Krefeld, Germany). The concentration of a given AtPIF3/6-EYFP variant in the lysate was determined by absorption measurements at 513 nm using a CLARIOstar microtiter plate reader (MTP) (BMG Labtech, Ortenberg, Germany). AtPhyB PCM at 2.5 µM concentration was mixed with a threefold molar excess of the AtPIF3/6-EYFP variants in 384-well clear MTPs (Thermo Fisher Scientific, Waltham, USA). After illumination with red light (650 ± 15 nm, 5.6 µW cm^{−2}) for 4 min, the MTPs were covered with a clear lid, and absorption at 720 and 850 nm was measured every 5 min at 28 °C in an Infinite M200 PRO plate reader (Tecan, Männedorf, Switzerland) for 12 h. After background correction, data at 720 nm were normalized to the signal of the L-EYFP (Supplementary Table 1) negative control, and the relative initial velocity was determined over the data acquired during the first 4 h.

Interaction assays with purified components. Size-exclusion chromatography. The light-dependent interaction between AtPhyB PCM and the AtPIF3/6-EYFP variants was assessed by gel filtration chromatography using a Superdex 200 Increase 10/300 GL (GE Healthcare) column on an ÄKTApure system, equipped with multi-wavelength detection (GE Healthcare). To this end, a mixture of 50 µM AtPhyB-PCM and 10 µM PIF-EYFP in 67 mM sodium phosphate buffer pH 8.0 and 200 mM NaCl was prepared and illuminated with 650- or 720-nm light for 2 min before sample application. Twenty-five microliters of this mixture was applied to the column and separated at a constant 0.75 mL min^{−1} flow rate. Absorption of EYFP and the AtPhyB PCM was measured at 513 and 650 nm, respectively. All proteins were also tested individually, where the AtPIF3/6-EYFP and EYFP samples were not illuminated prior to application.

Fluorescence anisotropy. AtPhyB PCM was illuminated with 640- or 750-nm light for 2 min immediately prior to the experiment (640 ± 15 nm; 65 µW cm^{−2} and 750 ± 15 nm; 420 µW cm^{−2}). Samples containing 20 nM AtPIF3/6-EYFP and increasing AtPhyB-PCM concentrations between 0 and 10 µM were prepared in 20 mM HEPES/HCl pH 7.3, 10 mM NaCl, and 100 µg mL^{−1} bovine serum albumin, transferred into black 384-well MTPs (Brand, Wertheim, Germany), and illuminated with 640- or 750-nm light. Fluorescence anisotropy of EYFP fluorophore was measured on a CLARIOstar MTP reader (BMG Labtech) with an excitation wavelength of 482 ± 16 nm, a 504-nm long-pass dichroic filter, and a detection wavelength of 530 ± 40 nm. The fluorescence gains for the horizontal and vertical detection channels were adjusted to a fluorescence anisotropy value of 0.315, as determined for EYFP with an Olis DSM 172 spectrophotometer (On-Line Instrument Systems, Bogart, USA). Anisotropy data were evaluated with the Fitomat software⁷³ using a single-site binding isotherm:

$$r = r_0 + r_1 \frac{[\text{PhyB}]}{[\text{PhyB}] + K_D}$$

where r represents the anisotropy of the PIF-EYFP fluorescence, $[\text{PhyB}]$ is the concentration of the AtPhyB PCM in either the Pr or Pfr state, and K_D is the dissociation constant. For the case of strong binding exhibited by the variants P6.100 and P6, we used a modified single-site binding isotherm that takes into account that the relevant $[\text{PhyB}]$ concentrations are on the same order of magnitude as the constant concentration c_{total} of the PIF-EYFP protein:

$$r = r_0 + r_1 / 2 \times \left\{ 1 + [\text{PhyB}] / c_{\text{total}} + K_D / c_{\text{total}} - \sqrt{(1 + [\text{PhyB}] / c_{\text{total}} + K_D / c_{\text{total}})^2 - 4[\text{PhyB}] / c_{\text{total}}} \right\}$$

Light-regulated gene expression in mammalian cells. The split transcription factor system for light-controlled gene expression in eukaryotic cells was based on a previously reported set-up^{36,44}. To allow ratiometric analysis, this earlier set-up

was expanded by cloning the *Gussia* luciferase under the control of a constitutive promoter onto the same plasmid as the SEAP reporter gene. For testing of the *AtPIF* variants, *AtPIF6* (1–100) was replaced by the corresponding *AtPIF1/3/6* derivatives. CHO-K1 (DSMZ, Braunschweig, Germany) were cultivated in HAM's F12 medium (PAN Biotech, Aidenbach, Germany; no. P04–14500) supplemented with 10% (v/v) tetracycline-free fetal bovine serum (PAN Biotech; no. P30–3602; batch no. P080317TC) and 1.4% (v/v) streptomycin (PAN Biotech; no. P06–07100). In all, 5×10^4 CHO-K1 cells were transfected using polyethylenimine (PEI; Polysciences Inc. Europe, Hirschberg, Germany; no. 23966–1)⁷⁴. DNA (0.75 µg) was diluted in 50 µL OptiMEM (Invitrogen, Thermo Fisher Scientific) and mixed with a PEI/OptiMEM mix (2.5 µL PEI solution in 50 µL OptiMEM). The DNA–PEI mix was added to the cells after 15 min of incubation at room temperature. At 4 h post-transfection, the medium was exchanged. CHO-K1 cells were transfected with the reporter plasmid *etr8-CMVmin-SEAP-BGH-SV40-Gussia* (pPF035) and the different *AtPhyB:AtPIF* variants. All plasmids were transfected in equal amounts (w/w). At 24 h post-transfection, the cells were supplemented with 15 µM phycocyanobilin (24 mM stock solution in DMSO; Frontier Scientific, Logan, UT, USA; no. P14137) and incubated for 1 h. The cells were then illuminated with 660-nm light for 24 h at an intensity of $20 \mu\text{mol m}^{-2} \text{s}^{-1}$, delivered by custom-built LED light boxes³⁶, or kept in darkness. As a negative control, the reporter construct alone was transfected. Photoreversibility was tested by incubating cells for 24 h under red light, followed by exchange of the media and incubation under far-red light for 24 h. In parallel, cells were incubated in darkness for 48 h with media exchange after 24 h. Exchange of media and other cell handling was done under 522-nm safe light, to prevent inadvertent actuation of the light-sensitive systems.

SEAP activity assay: The supernatant of transfected cells was transferred to 96-well round-bottom MTPs and incubated at 68 °C for 1 h to inactivate endogenous phosphatases. Afterwards, 80 µL of the supernatant were transferred to 96-well flat-bottom MTPs, and per well 100 µL SEAP buffer [20 mM homoarginine, 1 mM MgCl_2 , 21% (v/v) diethanolamine] was added³⁶. After addition of 20 µL 120 mM para-nitrophenyl phosphate, the absorption at 405 nm was measured for 1 h using a BMG Labtech CLARIOstar or a TriStar2 S LB 942 multimode plate reader (Berthold Technologies, Bad Wildbad, Germany)³⁶. Outliers were statistically determined and excluded⁷⁵.

Gussia luciferase assay: Twenty microliters of the supernatant of the transfected cells were transferred to a 96-well white MTP and diluted in 60 µL phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4 , 8.03 mM Na_2PO_4 , 137 mM NaCl). After addition of 20 µL coelenterazine (472 mM stock solution in methanol, diluted 1:1500 in PBS; Carl Roth, Karlsruhe, Germany, no. 4094.4), the luminescence was measured for 20 min using TriStar2 LB 941 or LB 942 multimode plate readers.

Light-mediated membrane recruitment in mammalian cells. For each *AtPIF6* variant tested, a lentiviral vector (pHR) was constructed containing a membrane-bound *AtPhyB* PCM (PHY-CAAX, residues 1–650) and a YFP-conjugated *AtPIF6* variant. An IRES was introduced between the two coding sequences to ensure regulation of dual expression. Lentivirus was created by transfecting HEK-293T cells with pHR constructs and harvesting filtered media 48 h post-transfection. Mouse fibroblasts (NIH-3T3) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum. Fibroblasts were treated with lentivirus containing the constructs of interest. For all fibroblast experiments, cells were cultured in a 96-well glass-bottomed plate. Wells were pretreated with fibronectin for 30 min, following which fibronectin was aspirated and cells were plated and spun down for 5 min at 800 rpm. Cells were plated in 96-well glass-bottom plates and allowed to adhere for at least 12 h. Imaging was performed using a $\times 60$ oil immersion objective (NA 1.4) on a Nikon TI Eclipse microscope with a CSU-X1 confocal spinning disk, an EM-CCD camera, and appropriate laser lines, dichroics, and filters. DMEM was supplemented with phycocyanobilin 30 min prior to the start of the experiment. Cells were exposed to infrared light followed by red light to cause membrane recruitment and the resulting change in cytoplasmic fluorescence was measured using ImageJ by selecting a cytoplasmic region and computing the average pixel intensity before and after photostimulation. The change in cytoplasmic YFP-PIF level was normalized to the total YFP-PIF fluorescence in the nucleus under infrared conditions, to normalize to total expression level differences caused by lentivirus. In these experiments, light was delivered through the microscope using a Mightex Polygon digital micromirror device (DMD), X-Cite XLED1 LED light sources at 635 ± 20 and 730 ± 20 nm, and a $\times 40$ objective lens. The duration of LED illumination was 1 min. To estimate the light dose delivered to the cell, we measured the light intensity using a Thorlabs power meter (PM100D) when the DMD was set to 100% transmission and obtained 100 µW for 635-nm light and 20 µW for 730-nm light, over a field of view of about 100 µm squared. For all experiments, we set the DMDs to 5% dithering (so each region was only illuminated for 5% of the time), translating into a final calculated intensity of 5 µW 635-nm light and 1 µW of 730-nm light. The light was delivered over an approximately $100 \mu\text{m} \times 100 \mu\text{m}$ field of view, leading to an overall LED power density of 50 mW cm^{-2} at 635 nm and 10 mW cm^{-2} at 730 nm. Notably, these values are slightly higher but of comparable magnitude to those used by Pathak et al. for the *AtPhyB:AtPIF3/6* system in the context of light-regulated gene expression⁷⁶.

Statistics and reproducibility. Data are reported as mean $\pm$ SD or as mean $\pm$ SEM of $n \geq 3$ biologically independent replicates. Details are specified in the legends to the figures and tables. All experiments could be reproduced with similar results.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data underlying Figs. 2–6 are available in Supplementary Data 1. All data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

D.G. designed and cloned the AtPIF variants, expressed them and AtPhyB, analyzed light-dependent interactions in lysate and for purified proteins, cloned the constructs for light-regulated gene expression, and analyzed data. P.F. performed and evaluated experiments on light-regulated gene expression. S.G.J. conducted and evaluated experiments on light-regulated membrane recruitment. J.R.W.K. cloned and expressed several AtPIF variants and analyzed their interactions with AtPhyB in lysate. L.-O.E. advised on experimental design. J.E.T. supervised experiments on light-regulated membrane recruitment. M.D.Z. conceived the project and designed and supervised research. A.M. conceived the project and designed and supervised research. D.G., P.F., M.D.Z., and A.M. wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to M.D.Z. or A.M.

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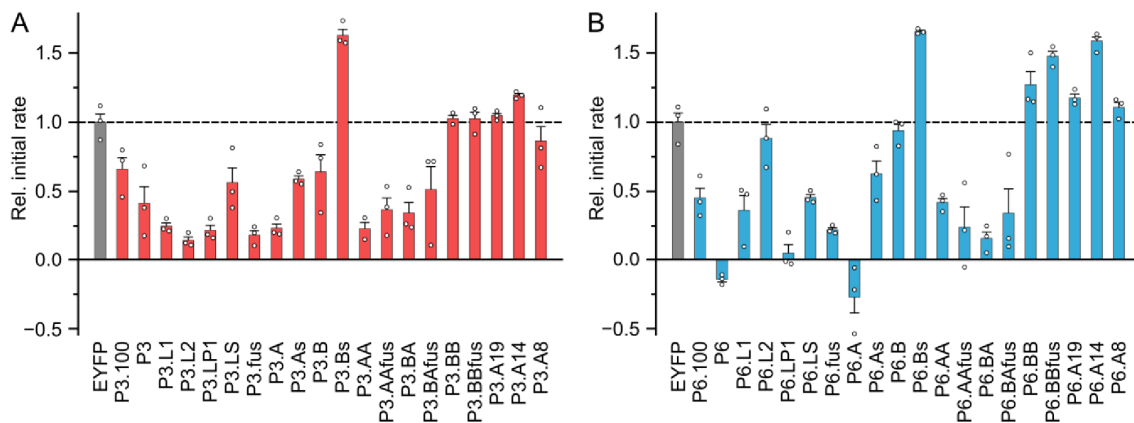
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Supplementary Figure 1

PIF1	70	-----PSMDPQQQSSD	CNLF	QED	MTSWLHYPLR	-----	100		
PIF2/PIL1	62	SLLDLYETEYSEGFKNI	-----	KILG	DTQVVPVSQSKPQ	QKET	100		
PIF3	60	RAREIGNGSKTTMVDEIPMSVPSIM	TGLS	QDD	EFPWLNHH	-----	100		
PIF4	58	EALR--SSTFLEDQETVSWIQYP	DEDP	EDF	SSHFFSTMDP	-----	100		
PIF5	60	ETLRKPNNIFLDNQETVQKPNYAAL	DDQ	-----	ETVSWIQYPPDDV	-----	100		
PIF6	46	SIMDLYEAEYNEDFMKSI	IHG	GGA	ITNLG	DTQVVPQSHVAAAHE	TNMLESNKHVD	100	
PIF7	37	QSL-----NGCETLESVVHQAAL	QPSK	FO	LS	PNGPNHNYESK	EGSCSRKRGYPQEMDRWFAVQ	EESH	100
PIF8	67	AGGTLESIVDQATRLPNPKPT	-----	DELVP	WFHHRSSR	-----	100		

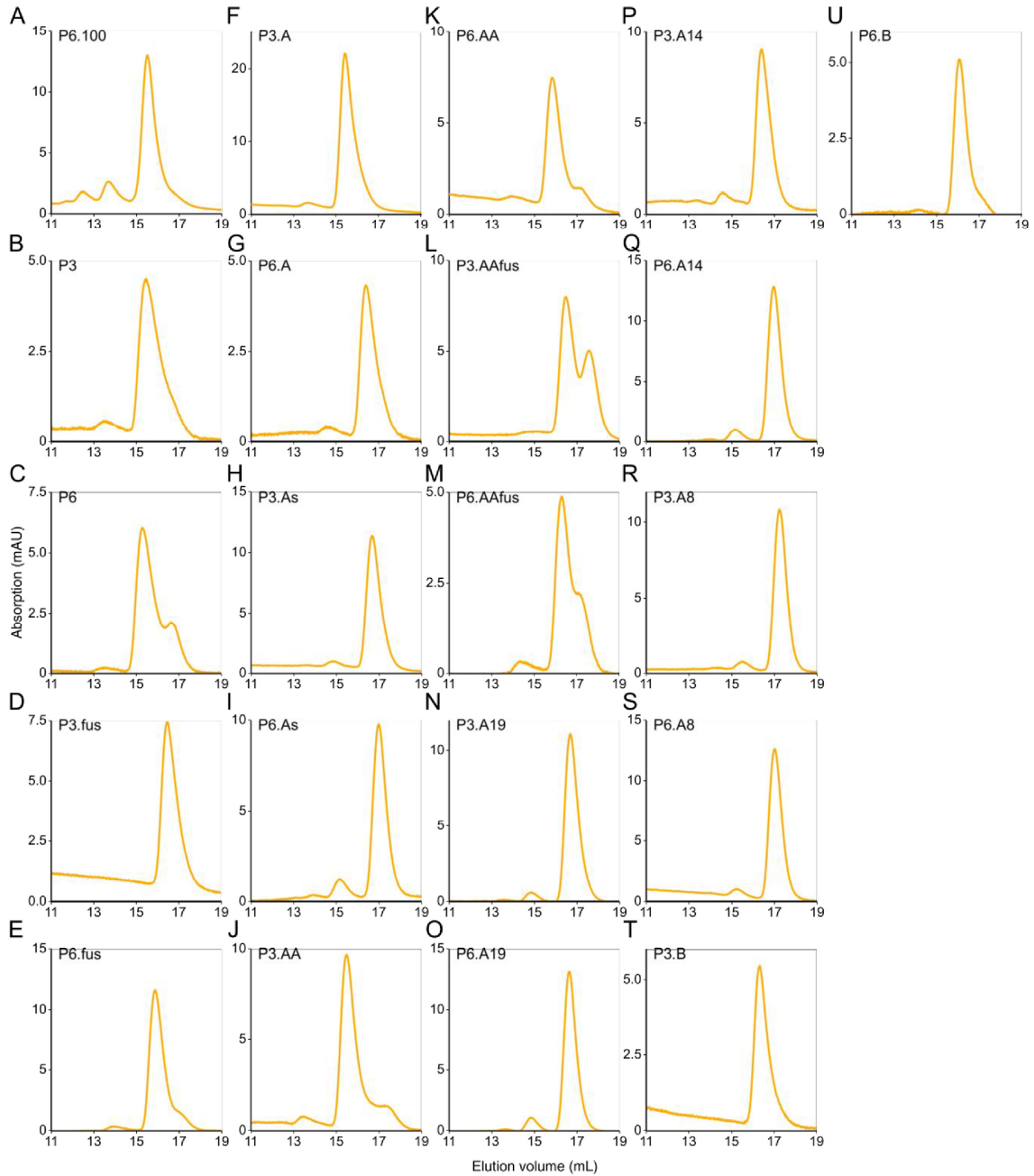
Sequence alignment of the N-terminal segments of the *A. thaliana* PIFs 1-8 according to Khanna *et al.*¹ Red color marks the N-terminal methionine; violet and gray color indicate strictly conserved and moderately conserved residues, respectively. Boxes highlight the conserved APB.A (red) and APB.B (blue) segments.

Supplementary Figure 2



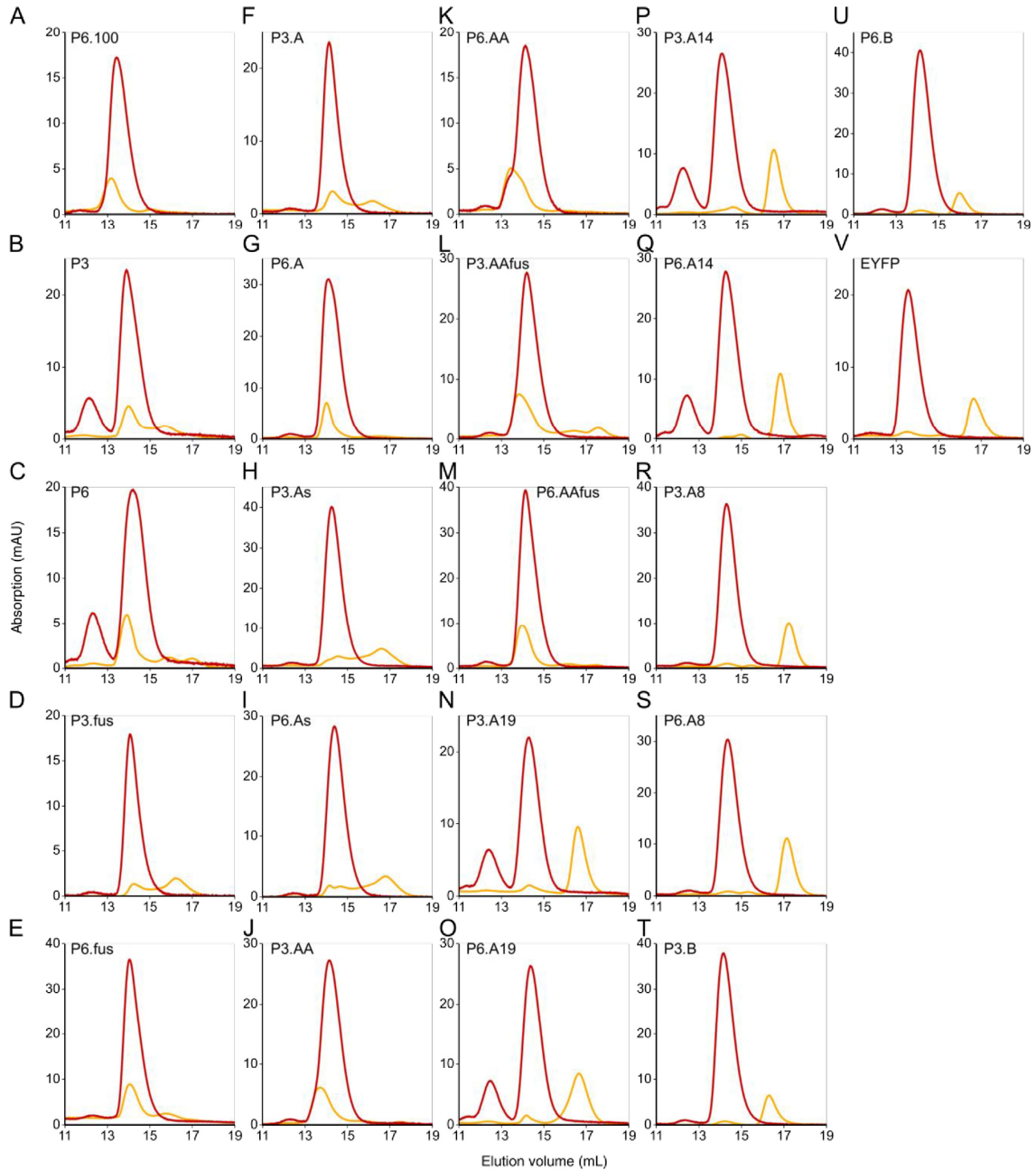
The initial rates of the recovery reaction of the AtPhyB PCM following red-light exposure were determined in bacterial lysate in the presence of different AtPIF variants and normalized to the reading obtained for the EYFP negative control. Data indicate mean $\pm$ SEM of $n = 3$ independent biological replicates. See Fig. 2 for details.

Supplementary Figure 3



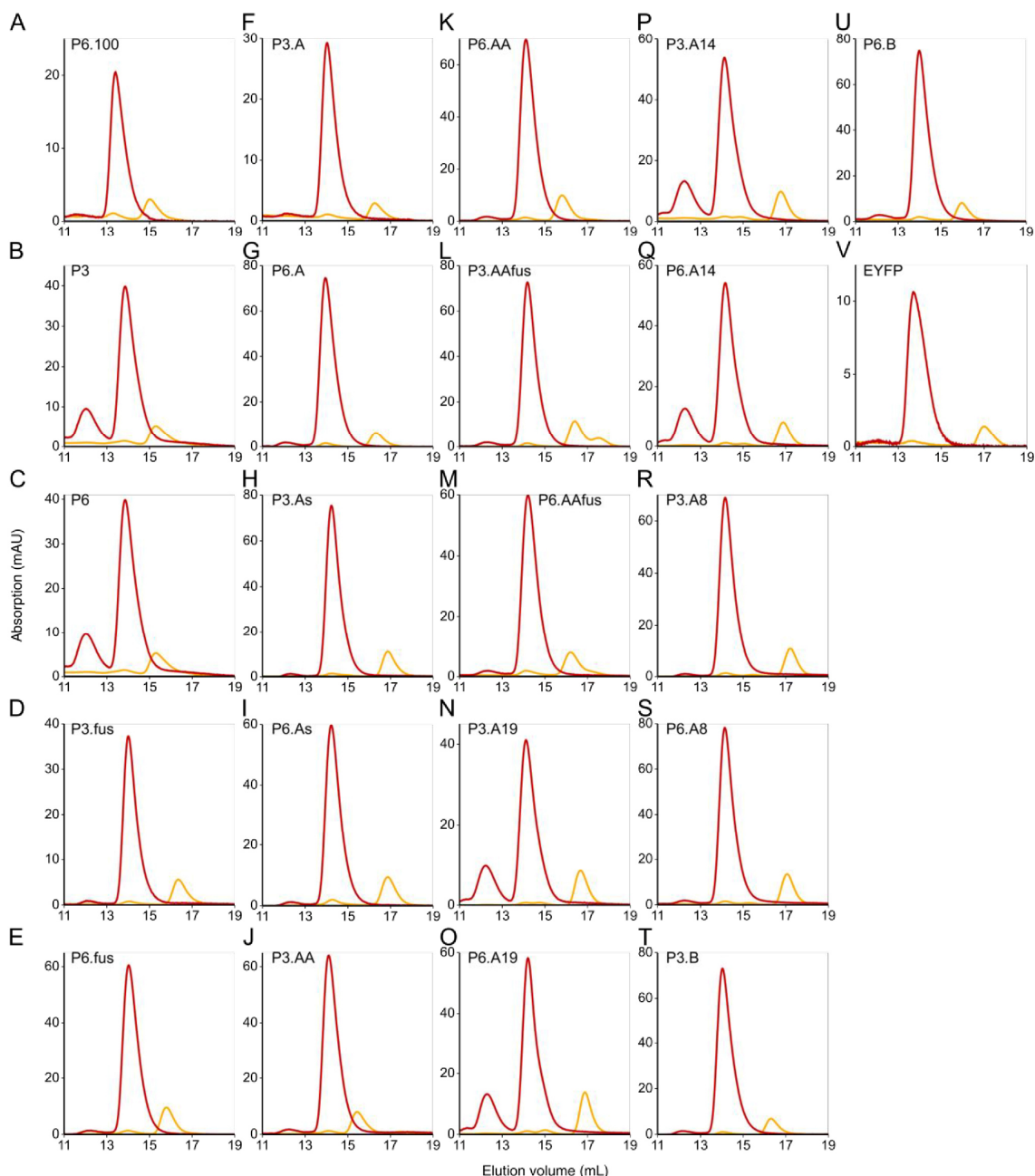
Oligomeric state of the AtPIF variants. (A) 10 μ M P6.100-EYFP were analyzed by size-exclusion chromatography, where the yellow lines represent the absorption at 513 nm. (B-U) As in (A), but for (B) P3; (C) P6; (D) P3.fus; (E) P6.fus; (F) P3.A; (G) P6.A; (H) P3.As; (I) P6.As; (J) P3.AA; (K) P6.AA; (L) P3.AAfus; (M) P6.AAfus; (N) P3.A19; (O) P6.A19; (P) P3.A14; (Q) P6.A14; (R) P3.A8; (S) P6.A8; (T) P3.B; (U) P6.B.

Supplementary Figure 4



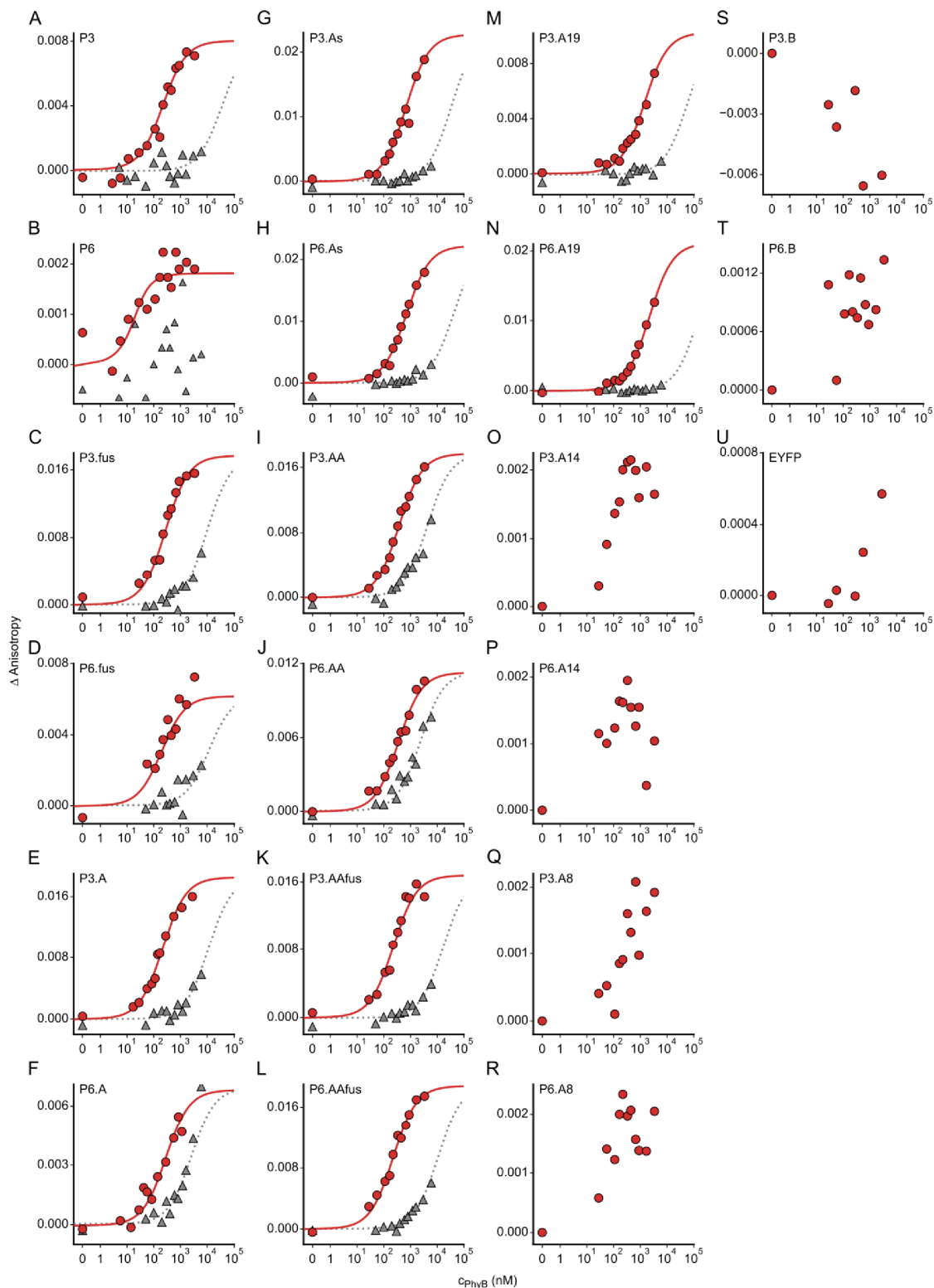
Light-dependent interactions of the AtPIF variants with the Pfr state of the AtPhyB PCM. (A) A mixture of 10 μ M P6.100-EYFP and 50 μ M AtPhyB PCM was exposed to red light and analyzed by size-exclusion chromatography, where the yellow and red lines represent the absorption at 513 and 650 nm, respectively. (B-V) As in (A), but instead of P6.100-EYFP for (B) P3; (C) P6; (D) P3.fus; (E) P6.fus; (F) P3.A; (G) P6.A; (H) P3.As; (I) P6.As; (J) P3.AA; (K) P6.AA; (L) P3.AAfus; (M) P6.AAfus; (N) P3.A19; (O) P6.A19; (P) P3.A14; (Q) P6.A14; (R) P3.A8; (S) P6.A8; (T) P3.B; (U) P6.B; (V) EYFP. The schematics in the graphs indicate the composition of the AtPIF variants, with variants deriving from AtPIF3 and AtPIF6 shown in red and blue, respectively.

Supplementary Figure 5



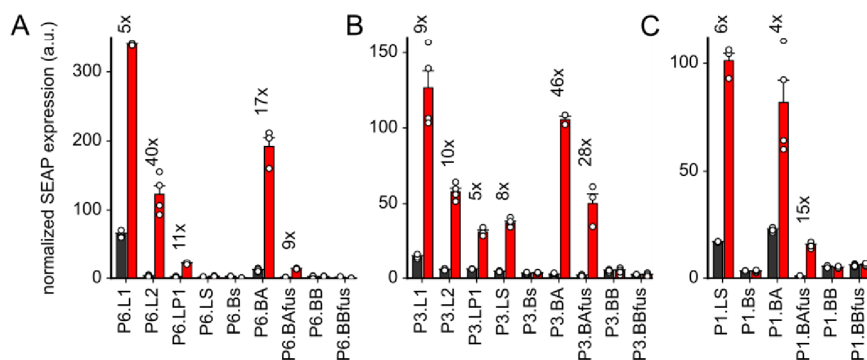
Light-dependent interactions of the AtPIF variants with the Pr state of the AtPhyB PCM. (A) A mixture of 10 μ M P6.100-EYFP and 50 μ M AtPhyB PCM was exposed to far-red light and analyzed by size-exclusion chromatography, where the yellow and red lines represent the absorption at 513 and 650 nm, respectively. (B-V) As in (A), but instead of P6.100-EYFP for (B) P3; (C) P6; (D) P3.fus; (E) P6.fus; (F) P3.A; (G) P6.A; (H) P3.As; (I) P6.As; (J) P3.AA; (K) P6.AA; (L) P3.AAfus; (M) P6.AAfus; (N) P3.A19; (O) P6.A19; (P) P3.A14; (Q) P6.A14; (R) P3.A8; (S) P6.A8; (T) P3.B; (U) P6.B; (V) EYFP. The schematics in the graphs indicate the composition of the AtPIF variants, with variants deriving from AtPIF3 and AtPIF6 shown in red and blue, respectively.

Supplementary Figure 6



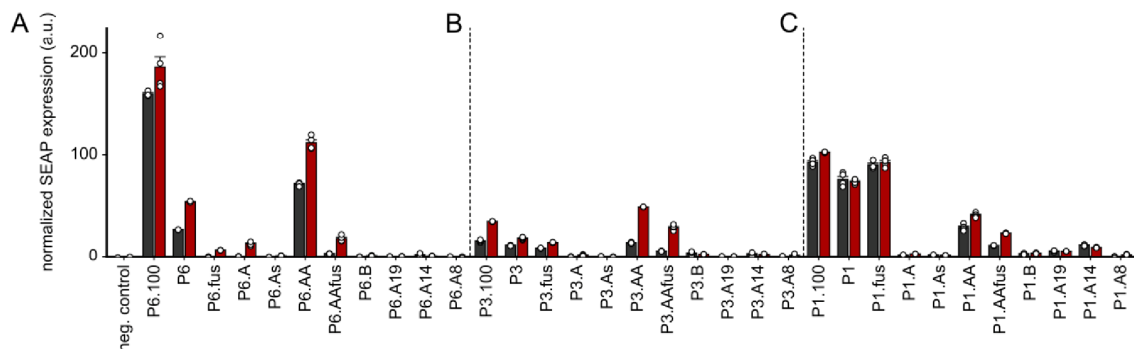
(gray) or red-light-exposed *AtPhyB* PCM (red), as monitored by anisotropy of the EYFP fluorescence. Data points show averages of three biological replicates. The lines denote fits to single-site binding isotherms. (B-U) As in (A), but instead of P3-EYFP for (B) P6; (C) P3.fus; (D) P6.fus; (E) P3.A; (F) P6.A; (G) P3.As; (H) P6.As; (I) P3.AA; (J) P6.AA; (K) P3.AAfus; (L) P6.AAfus; (M) P3.A19; (N) P6.A19; (O) P3.14; (P) P6.14; (Q) P3.8; (R) P6.8; (S) P3.B; (T) P6.B; (U) EYFP.

Supplementary Figure 7



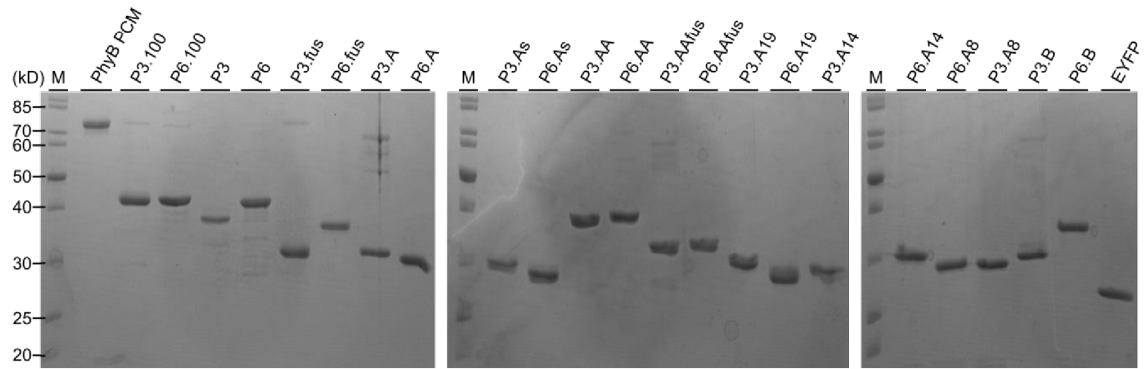
Harnessing the AtPIF variants for the light-dependent regulation of gene expression in mammalian cells. (A) SEAP expression was determined for the diverse AtPIF6 variants and normalized to the constitutive expression of *Gaussia* luciferase. Black and red bars denote mean normalized SEAP expression $\pm$ SEM for $n = 4$ independent biological replicates under dark conditions or red light, respectively. The numbers above the bars indicate the factor difference between dark and red-light conditions for a given AtPIF6 variant. (B) As panel (A) but for the AtPIF3 variants. (C) As panel (A) but for the AtPIF1 variants.

Supplementary Figure 8



Light-dependent regulation of gene expression in mammalian cells. The experiment was conducted as described in Fig. 5 but the cells were incubated in darkness for 48 h (black bars) or for 24 h under $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ 660-nm light, followed by $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ 740-nm light for another 24 h (brown). (A) SEAP expression was determined for the diverse *AtPIF6* variants and normalized to the constitutive expression of *Gussia* luciferase. Bars denote mean normalized SEAP expression $\pm$ SEM for $n = 4$ independent biological replicates. (B) As panel (A) but for the *AtPIF3* variants. (C) As panel (A) but for the *AtPIF1* variants.

Supplementary Figure 9



Analysis of the purified *AtPIF3/6*-EYFP proteins and the *AtPhyB* PCM by denaturing polyacrylamide gel electrophoresis.

Supplementary Tables

Supplementary Table 1. Amino-acid sequences of the *AtPIF* variants used in this study.

Name	Sequence ^a
P1.100	MHHFVPDFDT DDDYVNNHNS SLNHLPRKSI TTMGEDDDLM ELLWQNGQVV VQNQRLHTTK PSSSPKLLP SMDPQQQPSS DQNLFIQEDE MTSWLHYPLR
P3.100	MPLFELFRLT KAKLESAQDR NPSPPVDEVV ELVWENGQIS TQSQSSRSRN IPPPQANSSR AREIGNGSKT TMVDEIPMSV PSLMTGLSQD DDFVPWLNHH
P6.100	MMFLPTDYCC RLSDQEYMEI VFENGQILAK GQRSNVSLHN QRTKSIMDLTY EAEYNEDFMK SIIHGGGGAI TNLGDTQVVP QSHVAAAHET NMLESNKHVD
P1	MDDDLMEILLW QNGQVVVQNG RLHTKKPSSS PPKLLPCMDP QQQPSSDQNL FIQEDEMSTW LHYPLR
P3	MVDEVVEIVW ENGQISTQSQ SSRSRNIPPP QANSSRAREI GNGSKTTMVD EIPMSVPSLM TGLSQDDDFV PWNHH
P6	MDQEYMEIVF ENGQILAKGQ RSNVSLHNQR TKSIMDLTYA EYNEDFMKSI IHGGGGAITN LGDTQVVPQS HVAAAHETNM LESNKHVD
P3.L1	MVDEVVEIVW ENGQISTQSQ SSRSRRAREI GNGSKTTMVD EIPMSVPSLM TGLSQDDDFV PWNHH
P6.L1	MDQEYMEIVF ENGQILAKGQ RSNMDLYEAE YNEDFMKSII HGGGGAITNL GDTQVVPQSH VAAAHETNML ESNKHVD
P3.L2	MVDEVVEIVW ENGQISTQSQ SSRSRNIPPP QANSSRAREI GNGSKTTMTG LSQDDDFVPW LNHH
P6.L2	MDQEYMEIVF ENGQILAKGQ RSNVSLHNQR TKSIMDLTYA EYNEDAITNL GDTQVVPQSH VAAAHETNML ESNKHVD
P3.LP1	MVDEVVEIVW ENGQISTQSQ SSRSRKPSST PPKLLPCMDP QQQPSSDMTG LSQDDDFVPW LNHH
P6.LP1	MDQEYMEIVF ENGQILAKGQ RSNKPSSSPP KLLPCMDPQQ QPSSDAITNL GDTQVVPQSH VAAAHETNML ESNKHVD
P3.LS	MVDEVVEIVW ENGQISTQSQ SSRSRDSAGS AGSAGMTGLS QDDDFVPWLN HH
P6.LS	MDQEYMEIVF ENGQILAKGQ RSNDSAGSAG SAGAITNLGD TQVVPQSHVA AAHETNMLES NKHVD
P1.fus	MDDDLMEILLW QNGQVVVQNG RLHTKQNLFI QEDEMSTWLH YPLR
P3.fus	MVDEVVEIVW ENGQISTQSQ SSRSRMTGLS QDDDFVPWLN HH
P6.fus	MDQEYMEIVF ENGQILAKGQ RSNAITNLGD TQVVPQSHVA AAHETNMLES NKHVD
P1.A	MDDDLMEILLW QNGQVVVQNG RLHTKKPSSS PPKLLP
P3.A	MVDEVVEIVW ENGQISTQSQ SSRSRNIPPP QANSSRAREI GN
P6.A	MDQEYMEIVF ENGQILAKGQ RSNVSLHNQR TKSIMDLTYA
P1.B	MSMDPQQQPS SDQNLFIQED EMTSWLHYPL R
P3.B	MGSKTTMVDE IPMSVPSLMT GLSQDDDFVP WLNHH
P6.B	MEYNEDFMKS I IHGGGGAIT NLGDTQVVPQ SHVAAAHETN MLESNKHVD
P1.As	MDDDLMEILLW QNGQVVVQNG RLHTK

P3.As	MVDEVV	ELVW	ENGQ	ISTQSQ	SSRSR
P6.As	MDQEYM	ELVF	ENGQ	ILAKGQ	RSN
P1.Bs	MQNLFIQED	EMTSWLHYPL	R		
P3.Bs	MMT	GLSQDDDFVP	WLNHH		
P6.Bs	MAIT	NLGDTQVVPQ	SHVAAAHETN	MLESNKHVD	
P1.AA	MDDDLME	ELLW	QNGQ	VVVQNQ	RLHTKKPSSS PPKLLPCMDP QQQPSSDDDD
	LM	ELLWQNGQ	VVVQNQRLHT	KMTSWLHYPL	R
P3.AA	MVDEVV	ELVW	ENGQ	ISTQSQ	SSRSRNIPPP QANSSRAREI GNGSKTTMVD
	EIPMSVPSLV	DEVV	ELVWEN	GQISTQSQSS	RSRFVPWLNH H
P6.AA	MDQEYM	ELVF	ENGQ	ILAKGQ	RSNVSLHNQR TKSIMDLYEA EYNEDFMKSI
	IHGGGDQEY	MELVFENGQI	LAKGQRSNTN	MLESNKHVD	
P1.AAfus	MDDDLME	ELLW	QNGQ	VVVQNQ	RLHTKDDDLME ELWQNGQVV VQNQRLHTKM
	TSWLIHYPLR				
P3.AAfus	MVDEVV	ELVW	ENGQ	ISTQSQ	SSRSRVDEVV ELVWENGQIS TQSQSSRSRF
	VPWLNHH				
P6.AAfus	MDQEYM	ELVF	ENGQ	ILAKGQ	RSNDQEYMEL VFENGQILAK GQRSNTNMLE
	SNKHVD				
P1.BB	MQNLFIQEDE	KPSSSPKLL	PCMDPQQQPS	SDQNLFIQED	EMTSWLHYPL
	R				
P3.BB	MMTGLSQDDD	NIPPPQANSS	RAREIGNGSK	TTMVDEIPMS	VPSLMTGLSQ
	DDDFVPWLNH	H			
P6.BB	MAITNLGDTQ	VSLHNQRTKS	IMDLYEAEYN	EDFMKSIHG	GGGAITNLGD
	TQVVPQSHVA	AAHETNMLES	NKHVD		
P1.BBfus	MQNLFIQEDE	QNLFIQEDEM	TSWLHYPLR		
P3.BBfus	MMTGLSQDDD	MTGLSQDDDF	VPWLNHH		
P6.BBfus	MAITNLGDTQ	AITNLGDTQV	VPQSHVAAAH	ETNMLESNKH	VD
P1.BA	MQNLFIQEDE	KPSSSPKLL	PCMDPQQQPS	SDDDDLME	ELLWQNGQVVVNQ
	QRLHTKMTSW	LHYPLR			
P3.BA	MMTGLSQDDD	NIPPPQANSS	RAREIGNGSK	TTMVDEIPMS	VPSLVDEVVE
	LVWENGQIST	QSQSSRSRFV	PWLNHH		
P6.BA	MAITNLGDTQ	VVPQSHVAAA	HEVSLHNQRT	KSIMDLYEAE	YNEDFMKSI
	HGGGDQEYM	ELVFENGQIL	AKGQRSNTNM	LESNKHVD	
P1.BAfus	MQNLFIQEDE	DDDLME	ELLWQ	NGQVVVNQQR	LHTKMTSWLH YPLR
P3.BAfus	MMTGLSQDDD	VDEVV	ELVWE	NGQISTQSQS	SRSRFVPWLN HH
P6.BAfus	MAITNLGDTQ	VVPQSHVAAA	HEDQEYM	ELV	FENGQILAKG QRSNTNMLES
	NKHVD				
P1.19	MDDDLME	ELLW	QNGQ	VVVQNQ	
P3.19	MVDEVV	ELVW	ENGQ	ISTQSQ	
P6.19	MDQEYM	ELVF	ENGQ	ILAKGQ	
P1.14	MDDDLME	ELLW	QNGQ	V	
P3.14	MVDEVV	ELVW	ENGQ	I	

P6.14	MDQEYMEI ^{VF} EN ^{GQ} I
P1.8	ME ^{LL} WQNG ^Q
P3.8	ME ^{LV} WEN ^{GQ}
P6.8	ME ^{LV} FEN ^{GQ}

^a Red color marks the N-terminal methionine; violet and gray color indicates strictly conserved and moderately conserved residues, respectively.

Supplementary Reference

1. Khanna, R. et al. A Novel Molecular Recognition Motif Necessary for Targeting Photoactivated Phytochrome Signaling to Specific Basic Helix-Loop-Helix Transcription Factors. *Plant Cell* **16**, 3033–3044 (2004).

5.2 Manuskript II

Titel: „The association kinetics encode the light dependence of *Arabidopsis* phytochrome B interactions”

Autoren: Golonka, D.; Gerken, U.; Köhler, J.; Möglich, A.

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The Association Kinetics Encode the Light Dependence of *Arabidopsis* Phytochrome B Interactions

David Golonka^{1,†}, Uwe Gerken^{2,‡}, Jürgen Köhler^{2,3,4,*‡}, Andreas Möglich^{1,5,6,*‡}

¹Lehrstuhl für Biochemie, Photobiochemie, Universität Bayreuth, 95447 Bayreuth, Germany.

²Lehrstuhl für Spektroskopie weicher Materie, Universität Bayreuth, 95447 Bayreuth, Germany.

³Bayerisches Polymer Institut, Universität Bayreuth, 95447 Bayreuth, Germany.

⁴Bayreuther Institut für Makromolekülforschung, 95447 Bayreuth, Germany.

⁵Bayreuth Center for Biochemistry & Molecular Biology, Universität Bayreuth, 95447 Bayreuth, Germany.

⁶North-Bavarian NMR Center, Universität Bayreuth, 95447 Bayreuth, Germany.

*to whom correspondence may be addressed: juergen.koehler@uni-bayreuth.de,
andreas.moeglich@uni-bayreuth.de

†ORCID identifiers: D.G. 0000-0003-4101-4591; U.G. 0000-0002-6447-2803; J.K. 0000-0002-4214-4008; A.M. 0000-0002-7382-2772

Abstract

Plant phytochromes enable vital adaptations to red and far-red light. At the molecular level, these responses are mediated by light-regulated interactions between phytochromes and partner proteins, foremost the phytochrome-interacting factors (PIF). Although known for decades, quantitative analyses of these interactions have long been sparse. To address this deficit, we here studied by an integrated fluorescence-spectroscopic approach the equilibrium and kinetics of *Arabidopsis thaliana* phytochrome B (AtPhyB) binding to a tetramerized PIF6 variant. Several readouts consistently showed the stringently light-regulated interaction to be little affected by PIF tetramerization. Analysis of the binding kinetics allowed the determination of bimolecular association and unimolecular dissociation rate constants as a function of light. Unexpectedly, the elevated affinity of AtPhyB under red light relative to far-red light is entirely due to accelerated association rather than decelerated dissociation. The association reaction under red light is highly efficient and only tenfold slower than the diffusion limit. The present findings pertain equally to the analysis of signal transduction in plants and to the biotechnological application of phytochromes.

Keywords

binding; fluorescence spectroscopy; kinetics; optogenetics; phytochrome; protein-protein interaction; sensory photoreceptor; signal transduction

Introduction

Across many organisms, sensory photoreceptors mediate vital light-dependent adaptations of physiology, growth, development and motion [1,2]. Absorption of a photon by a chromophore embedded within the photosensor module of the receptor drives a cyclic series of photochemical reactions and conformational transitions, denoted the photocycle. Progression through the photocycle leads to population of the metastable signaling state of the receptor which differs in the biological activity of its effector module from that of the dark-adapted, resting state. Phytochromes (Phy) were identified in plants as the photoreceptors responsible for the ratiometric sensing of red and far-red light [3], and the regulation of essential processes including photomorphogenesis and shade avoidance [4–6]. Phys generally comprise an N-terminal photosensory core module (PCM) and a C-terminal effector or output module (OPM). The PCM consists of PAS (Per-ARNT-Sim) [7], GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) [8] and PHY (phytochrome-specific) domains [9–11], with a linear tetrapyrrole (bilin) chromophore nestled within the GAF domain. Plant Phys additionally possess an N-terminal extension (NTE) preceding the PCM. In darkness, conventional Phys populate their red-absorbing Pr state which is characterized by a Z configuration of the C15=C16 double bond of the bilin. Light absorption promotes population of the short-lived lumi-R and meta-R intermediates, followed by formation of the metastable Pfr state with the bilin in the 15E configuration [9]. The Pfr state passively reverts to Pr in a slow thermal reaction, or it can be actively returned to Pr via absorption of a second photon of far-red color. The Pr $\leftrightarrow$ Pfr transition couples to the refolding of a protrusion of the PHY domain, the so-called PHY tongue, from a β -hairpin to an α -helix conformation [12–14]. In turn, larger-scale structural changes are elicited that underpin signal propagation and regulation of the OPM.

In case of plant Phys, the OPM consists of two PAS domains, PAS-A and PAS-B, and a histidine-kinase-related domain (HKRD), reported to harbor serine/threonine kinase activity [15] (Fig. 1a). Even in the absence of the OPM [16–19], plant Phys enter light-dependent protein:protein interactions (PPI) with partner proteins, in particular the phytochrome-interacting factors (PIF), a family of transcription factors. *Arabidopsis thaliana* possesses five phytochromes (AtPhyA–AtPhyE) and at least eight PIF proteins, termed AtPIF1–8 [4,16,20–23]. The N-terminal AtPIF segments of around 100 amino acids comprise a weakly conserved, bipartite motif denoted APB (active phytochrome B binding) that subdivides into its constituent APB.A and APB.B parts and is responsible for light-dependent PPIs with AtPhyB (Fig. 1a). The C-terminal AtPIF segments harbor basic helix-loop-helix modules capable of DNA binding. Within the plant cell, the light-dependent PPIs influence the nucleocytoplasmic distribution of the AtPhy and AtPIF proteins, and their cellular activity and stability [4,6,23,24], which together underlie diverse biological responses to red/far-red light. The light-dependent AtPhy:AtPIF interaction has also been exploited for biotechnology to bring into spatial proximity target proteins in response to light cues. Compared to constitutive or chemically inducible dimerization systems, light-dependent PPIs afford the advantages of i. high spatial and temporal precision; ii. reversibility; and iii. repeated activation of the system. Within the

field of optogenetics [25], plant Phys have been used to regulate by red/far-red light gene expression [19,26–28], PPIs and intracellular localization [26,29–31].

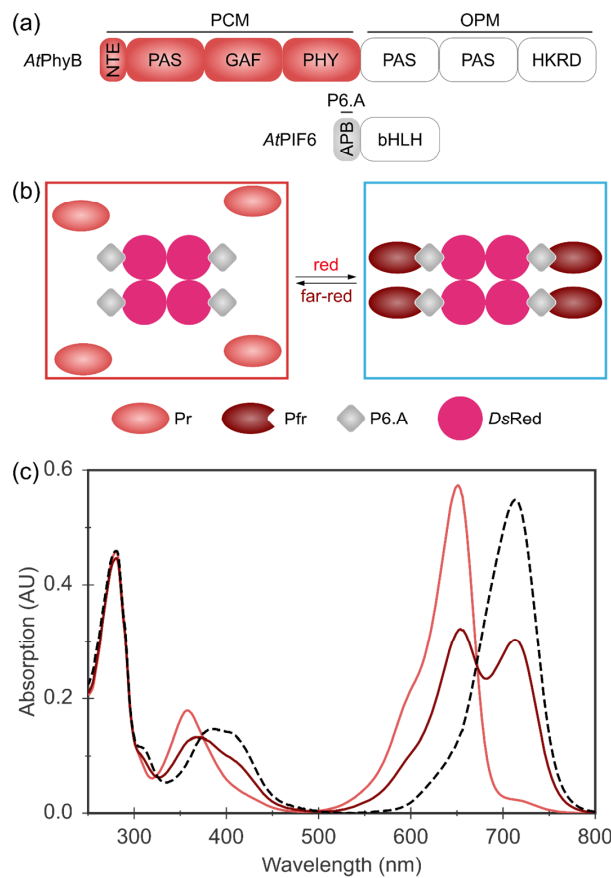


Figure 1: Architecture and function of plant phytochromes (Phy) and the phytochrome-interacting factors (PIFs). (a) Modular composition of *Arabidopsis thaliana* PhyB and PIF6. AtPhyB comprises an N-terminal extension (NTE), a photosensory core module (PCM) (red) and an output module (OPM). AtPIF6 possesses an active phytochrome B binding (APB) motif (gray) responsible for interaction with AtPhyB, succeeded by a basic-helix-loop-helix (bHLH) motif capable of DNA binding. P6.A denotes an APB fragment of 40 residues in size necessary and sufficient for light-dependent interaction with AtPhyB [32]. (b) P6.A (gray) is fused to the homotetrameric fluorescent protein DsRed-Express2 (magenta circle). In its dark-adapted Pr state (red ellipsoid), the AtPhyB PCM does not interact with P6.A-DsRed. Upon red-light exposure, the AtPhyB PCM populates its Pfr state and interacts with P6.A-DsRed. (c) Absorption spectra of the AtPhyB PCM. Following exposure to far-red light (brown line), the protein is almost entirely within its red-absorbing Pr state. Upon red-light exposure (red line), a photostationary Pr:Pfr mixture is assumed. The dashed line shows the calculated spectrum of the pure Pfr state.

Notwithstanding the prime relevance of plant Phy-mediated PPIs in nature and biotechnology, quantitative data on the interaction under red and far-red light have long been lacking. Recently, we assessed by fluorescence anisotropy the interactions of AtPIF3 and AtPIF6 with the AtPhyB PCM in both its Pr and Pfr states [32]. We not only confirmed that the N-terminal AtPIF fragments (residues 1-100) suffice for red-light-activated PPIs with the AtPhyB PCM [16] but also put concrete numbers to these interactions. For AtPIF6, the affinity to the Pfr state of AtPhyB was around 10 nM

but that to the Pr state was weaker than 10 μ M. By contrast, AtPIF3 exhibited reduced Pfr-state and elevated Pr-state affinities. Whereas the N-terminal APB.A part of the APB motif dominated the light-dependent PPI, the C-terminal APB.B part contributed to and fine-tuned the response. Through omission of the APB.B part and truncation of flanking regions, we constructed a minimized AtPIF6 fragment, denoted P6.A (Fig. 1a), of 40 residues in size, yet retaining high affinity for the Pfr state of the AtPhyB PCM (about 280 nM) and low affinity for the Pr state ($> 2 \mu$ M). Moreover, P6.A adopted monomeric state in solution, whereas the more extended AtPIF variants were predominantly or exclusively homodimeric.

Here, we capitalized on the monomeric state of P6.A and coupled it to the homotetrameric fluorescent protein DsRed (Fig. 1b). Doing so enabled the characterization of the light-dependent association equilibrium and kinetics of the interaction with the AtPhyB PCM by a multimodal fluorescence-spectroscopic approach, encompassing intensity, anisotropy, lifetime, correlation and energy transfer measurements. Tetramerization of P6.A via the DsRed protein did not greatly affect the photoreversible association equilibrium compared to monomeric P6.A, nor did it entail cooperativity of binding. The integrated fluorescent analyses revealed that the bimolecular association reaction of the AtPhyB:P6.A pair is highly efficient in the Pfr state, with kinetics only around 10-fold slower than the diffusion limit. Remarkably, the differential affinity of the Pr and Pfr states is entirely down to the association reaction, rather than the dissociation.

Results

Light-dependent Interaction between Tetramerized PIF and Phytochrome

As shown in multiple previous studies [16–19,26,27,32], the NTE and the PAS-GAF-PHY PCM of AtPhyB are both necessary and sufficient for light-dependent PPIs with AtPIF proteins. Although shorter AtPhyB fragments solely consisting of the NTE and the PAS and GAF domains exhibited light-dependent responses *in planta* [33], their photoreversible interaction with AtPIF proteins appears impaired [26]. Moreover, as the PHY domain stabilizes the Pfr state through direct interactions with the bilin chromophore [9,13,34], it is required for efficient photoconversion and population of Pfr. As before [32], we hence used in our analysis an N-terminal AtPhyB fragment comprising the NTE and the intact PCM with its PAS, GAF and PHY domains (residues 1-651). In the following, we refer to this construct as AtPhyB PCM. While full-length AtPhyB is homodimeric, serial C-terminal truncations of its HKRD, PAS-A and PAS-B domains successively render it monomeric [35]. We previously demonstrated by size-exclusion chromatography that the present AtPhyB PCM construct is predominantly monomeric up to concentrations of at least 50 μ M [32]. In darkness, the AtPhyB PCM adopts the Pr state (Fig. 1c); illumination with red light (650 nm) drives the Pr $\rightarrow$ Pfr conversion but simultaneously also the reverse Pfr $\rightarrow$ Pr reaction, thus giving rise to a photostationary state with a mixed Pr/Pfr population at around 0.44/0.56 ratio (Fig. 1c). Far-red light (720 nm) returns the AtPhyB PCM to the Pr state, with only a minor Pfr fraction of about 0.02 remaining.

Our previous investigation had identified the shortened AtPIF6 variant P6.A (residues 14-53) that retains light-dependent interactions with the AtPhyB PCM but is monomeric in contrast to longer PIF constructs [32]. The monomeric state of P6.A benefits its coupling to oligomeric scaffolds which is of considerable interest for applications in optogenetics and biotechnology [36]. To assess the AtPIF:AtPhy interaction within an oligomeric context and to provide hitherto unavailable information on association and dissociation kinetics, we N-terminally connected P6.A to the homotetrameric fluorescent protein DsRed-Express 2 (shorthand DsRed in the following) [37] (Fig. 1b). Fusion to P6.A did not alter the spectral properties of the fluorescent protein. Notably, the DsRed emission spectrum is red-shifted compared to that of EYFP used in our previous study [32], thus resulting in more extensive overlap with the absorption spectrum of the interacting Pfr state of the AtPhyB PCM (Suppl. Fig. S1). Assuming isotropically arranged and rapidly reorienting point dipoles, we calculate a characteristic distance R_0 of 41 Å for resonance energy transfer from DsRed to AtPhyB Pfr [38,39]. When incubated with excess AtPhyB PCM, the fluorescence intensity of P6.A-DsRed was reduced by around 14% following red-light exposure compared to far-red-light exposure (Suppl. Fig. S2a). This change corresponds to a drop of fluorescence quantum yield from 0.42 for free P6.A-DsRed (assumed to equal the value for DsRed-Express2 [34]) to around 0.36 when interacting with the Pfr state of AtPhyB PCM and indicates energy transfer between the two proteins. We harnessed this effect to analyze the light-dependent PPI between P6.A-DsRed and increasing concentrations of AtPhyB PCM (Suppl. Fig. S2). To correct for deviations in sample concentration, we divided the DsRed fluorescence readings after exposure to red light by those after exposure to far-red light. The normalized fluorescence monotonically decreased with increasing AtPhyB PCM and could be described by a binding isotherm. Assuming identical and non-interacting binding sites within the tetrameric P6.A-DsRed, we obtained an apparent dissociation constant K_d of (50 ± 15) nM. As the simple isotherm accounts for the experimental data, more complex models are not justified. Owing to the normalization of the fluorescence intensity data, the K_d value mainly reflects interactions of the Pfr state of AtPhyB PCM. By contrast, reliable estimates on the Pr-state affinity could not be obtained by this approach.

To remedy this shortcoming, we next analyzed the light-regulated PPI by fluorescence anisotropy of P6.A-DsRed, which to first approximation is insensitive to deviations in sample concentration (Suppl. Fig. S3a). Binding of AtPhyB PCM to P6.A-DsRed is expected to increase its hydrodynamic radius, resulting in a slowing of rotational diffusion and concomitant rise of anisotropy. The observed anisotropy increase with AtPhyB PCM concentration could be described by a simple binding isotherm with K_d values of (50 ± 10) nM and $> 1 \mu\text{M}$ for the Pfr and Pr states of AtPhyB PCM, respectively. As noted above, red light drives both the Pr→Pfr and the Pfr→Pr transitions in AtPhyB, thus leading to a 0.44/0.56 Pr/Pfr photostationary mixture (Fig. 1c). Hence, here and in the following the K_d value obtained upon red-light exposure was adjusted by a factor of 0.56 and refers to the concentration of AtPhyB PCM in the Pfr state. We previously determined values for the interaction between monomeric EYFP-labeled P6.A and the AtPhyB PCM of (280 ± 10) nM in Pfr and weaker than $2 \mu\text{M}$ in Pr [32]. We note that the somewhat lower K_d value obtained

presently can be attributed to the simple binding isotherm which effectively considers only unbound and fully bound P6.A-*DsRed* but not partially bound species. Although more complex models could potentially address this deficit, these are not warranted by the data and would invariably require prior assumptions about the system. We hence stayed with the simplest binding model but caution that the determined K_d should be considered an apparent value. To assess the P6.A-*DsRed*:*AtPhyB* PCM interaction in more detail, we recorded fluorescence anisotropy as a function of addition of red-light-exposed *AtPhyB* PCM in the regime of strong binding at elevated P6.A-*DsRed* concentrations well above the K_d value (Suppl. Fig. S3b). We normalized the observed anisotropy to unity and plotted it against the molar ratio of *AtPhyB* PCM:P6.A-*DsRed*. At low ratios, the anisotropy increased with a slope of about 4 ± 1 which indicates that in this regime one molecule *AtPhyB* PCM interacts with approximately four molecules P6.A-*DsRed*, i.e. one homotetramer. The anisotropy levels off at around a ratio of 1, indicating that at saturation four molecules *AtPhyB* PCM bind one P6.A-*DsRed* homotetramer.

Multimodal Fluorescence Analysis of the Light-dependent Binding Equilibrium

To probe the hydrodynamic behavior of the *AtPhyB* PCM and P6.A-*DsRed*, and to further analyze their light-dependent interaction, we resorted to fluorescence correlation spectroscopy (FCS) (Fig. 2a). This approach is predicated on the increase in hydrodynamic radius expected for P6.A-*DsRed* upon binding up to four molecules *AtPhyB* PCM, corresponding to a 2.5-fold increase of molecular weight. The resultant slowdown of transversal diffusion gives rise to an increased correlation time in FCS measurements. We first performed FCS on 100 nM *AtPhyB* PCM which in its Pr state weakly fluoresces with an emission maximum at around 670 nm (Suppl. Fig. S4a) [40]. From the FCS data, we determined a transversal diffusion coefficient D of $(65 \pm 1) \mu\text{m}^2 \text{s}^{-1}$ (Table 1). Approximating the *AtPhyB* PCM as a spherical particle and using the Stokes-Einstein equation, this yields a hydrodynamic radius R_H of $(3.3 \pm 0.1) \text{ nm}$, broadly consistent with the known monomer dimensions [34].

Tabelle 1: Diffusion coefficients D and Stokes radii R_H

Protein(s)	$D (\mu\text{m}^2 \text{s}^{-1})$ ^b	$R_H (\text{nm})$ ^c
<i>AtPhyB</i> PCM	65 ± 1	3.3 ± 0.1
<i>DsRed</i> -Express2	70 ± 2	3.1 ± 0.1
P6.A- <i>DsRed</i>	59 ± 1	3.6 ± 0.1
P6.A- <i>DsRed</i> + <i>AtPhyB</i> PCM ^a	49 ± 2	4.4 ± 0.2

^a The values refer to the complex formed between P6.A-*DsRed* and the *AtPhyB* PCM in its Pfr state upon red-light exposure.

^b As determined by fluorescence correlation spectroscopy.

^c As calculated from D according to the Stokes-Einstein relation.

For fluorescence correlation spectroscopy on P6.A-*DsRed*, we used a home-built setup with a 510-nm excitation laser that directly provides the autocorrelation function of photons emitted between 526 and 622 nm (Suppl. Fig. S4a). Of key advantage, the setup allows the simultaneous illumination of the sample with far-red light (720 nm) during the FCS experiment (Fig. 2a-b). For

reference, we first conducted measurements on 10 nM *DsRed* (Suppl. Fig. S5a-b) and observed that the correlation time τ_D linearly decreased with laser excitation intensity (Suppl. Fig. S4b) [41,42]. At an intensity of 2.0 kW cm⁻² used for all subsequent experiments, the correlation time amounted to around 185-190 μ s, independent of illumination with red or far-red light, and regardless of whether 700 nM *AtPhyB* PCM was added (Fig. 2c-d). These values correspond to a transversal diffusion coefficient D of $(70 \pm 2) \mu\text{m}^2 \text{s}^{-1}$ and a hydrodynamic radius R_H of $(3.1 \pm 0.1) \text{ nm}$ (Table 1), again in agreement with the known molecular structure of the homotetrameric protein [37]. We next studied P6.A-*DsRed* at 10 nM concentration and found a single correlation time τ_D of around 220 μ s, within error independent of illumination with red or far-red light (Fig. 2c-d). The higher value compared to that for *DsRed* reflects the appendage of the P6.A segment and corresponds to $D = (59 \pm 1) \mu\text{m}^2 \text{s}^{-1}$ and $R_H = (3.6 \pm 0.1) \text{ nm}$ (Table 1). In the presence of excess *AtPhyB* PCM (700 nM) and after illumination with red light, the correlation time τ_D increased to about 265 μ s (Figs. 2c-d), corresponding to $D = (49 \pm 2) \mu\text{m}^2 \text{s}^{-1}$ and $R_H = (4.4 \pm 0.2) \text{ nm}$ (Table 1). When the experiment was conducted under simultaneous illumination with far-red light (720 nm) that drives the Pfr $\rightarrow$ Pr transition, τ_D assumed a value of about 225 μ s, closely similar to when no *AtPhyB* PCM was added. Taken together, FCS hence reveals the light-dependent PPI between P6.A-*DsRed* and the *AtPhyB* PCM. When FCS was performed on samples pre-conditioned by far-red light prior to the measurement but without far-red illumination during the measurement, the *AtPhyB* PCM was gradually photoactivated by the 510-nm excitation laser, thus triggering the interaction with P6.A-*DsRed* (Suppl. Fig. S5c).

We next exposed the P6.A-*DsRed*:*AtPhyB* PCM mixture to alternating cycles of red (650 nm) and far-red light (720 nm), and recorded FCS data (Fig. 3a). Over 12 cycles, the correlation times changed back and forth between values of $(259 \pm 9) \mu$ s under red light and $(206 \pm 5) \mu$ s under far-red light, indicating that the underlying PPI can be photoreversibly and repeatedly toggled in both directions. We next recorded FCS data for P6.A-*DsRed* in the presence of increasing *AtPhyB* PCM concentrations up to 1000 nM (Fig. 3b). Under red light, the correlation time increased hyperbolically with *AtPhyB* PCM and could be described by a single-site isotherm. Adjusted for the fraction 0.56 of *AtPhyB* PCM in the Pfr state, a dissociation constant of $(180 \pm 60) \text{ nM}$ results. Strikingly, no interaction could be detected for far-red-illuminated *AtPhyB* PCM even at 1 μ M concentration, which contrasts with the fluorescence anisotropy data (see above). We attribute this difference to the additional far-red illumination throughout the FCS measurement which constantly reverts minor fractions of Pfr that may be present at equilibrium or accumulate during the experiment to the Pr state.

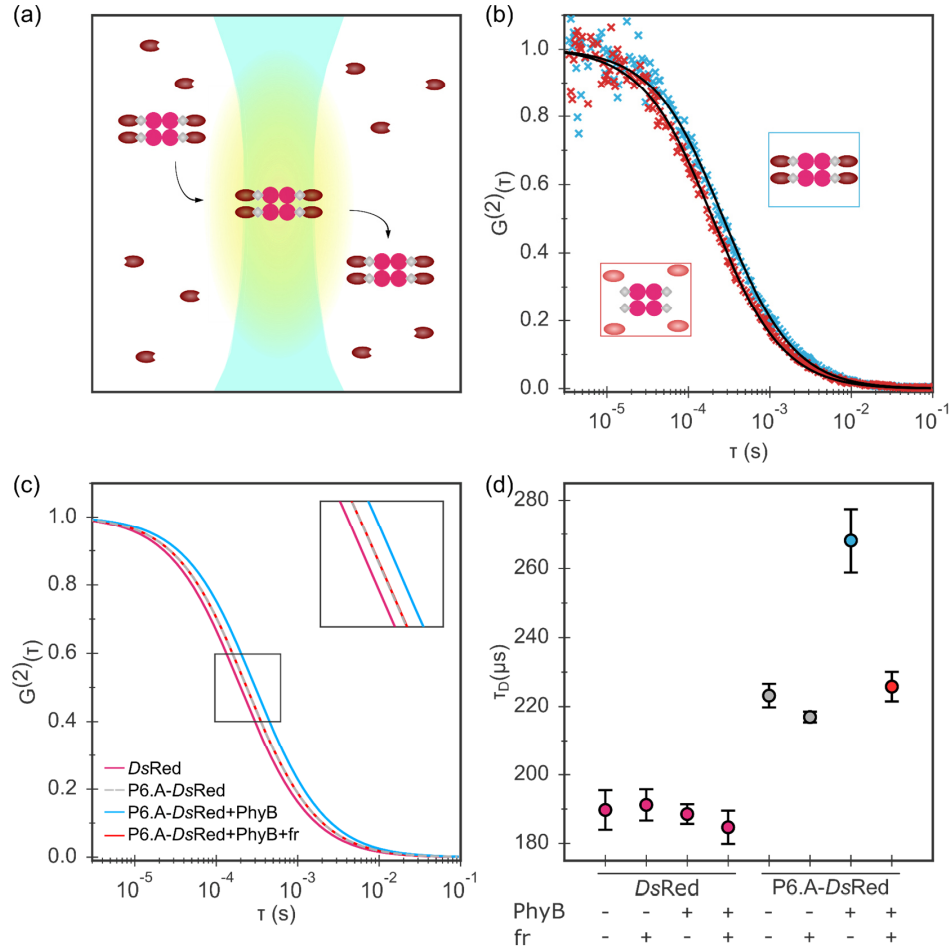


Figure 2: Fluorescence correlation spectroscopy (FCS) of the PhyB:PIF interaction. (a) Schematic representation of the FCS experiment. Upon red-light exposure, the AtPhyB PCM is present in its Pfr state and binds to P6.A-DsRed, which increases the size of the fluorescent particles. (b) Normalized autocorrelation function for P6.A-DsRed under far-red light (720 nm, red symbols) or after red-light exposure (650 nm, blue symbols), and in the presence of 700 nM AtPhyB PCM. Black curves represent fits to a standard three-dimensional diffusion model (eq. (7)). (c) Fitted autocorrelation functions for DsRed (magenta), P6.A-DsRed (gray dashed), and a mixture of P6.A-DsRed and 700 nM AtPhyB PCM under far-red (red) and red light (blue). (d) Correlation times τ_D for DsRed (magenta) or P6.A-DsRed (gray) with or without AtPhyB PCM, either under far-red (fr) light (red) or red light (blue). Data points show the average of at least three independent measurements.

As the FCS setup records the emission from the sample in time-resolved manner, we also analyzed the P6.A-DsRed fluorescence lifetimes τ as a function of lighting conditions and AtPhyB PCM concentration. In the absence of the AtPhyB PCM, the fluorescence decayed in a biexponential manner with lifetimes τ_1 and τ_2 of (1.19 ± 0.04) ns and (2.64 ± 0.02) ns and associated amplitudes of (17.1 ± 0.1) % and (82.9 ± 0.1) % (Suppl. Fig. S4c-d). Biexponential fluorescence decay kinetics have been reported for DsRed before [43–45]. When exposed to far-red light, the addition of AtPhyB PCM had no significant influence on either time constant. By contrast, after red-light illumination, the slower lifetime component τ_2 decreased with AtPhyB PCM concentration in hyperbolic manner to (2.45 ± 0.01) ns at 1000 nM AtPhyB PCM, whereas the faster lifetime component stayed constant

within error (Suppl. Fig. S5d). The observed decrease in τ_2 by about 7% is ascribed to energy transfer from P6.A-*DsRed* to the Pfr state of the *AtPhyB* PCM, consistent with the static analysis of fluorescence emission above (see Suppl. Fig. S2). A single-site binding isotherm describes the concentration-dependent decrease of τ_2 and yields a dissociation constant of (300 ± 80) nM, similar to the value determined from the FCS τ_D values.

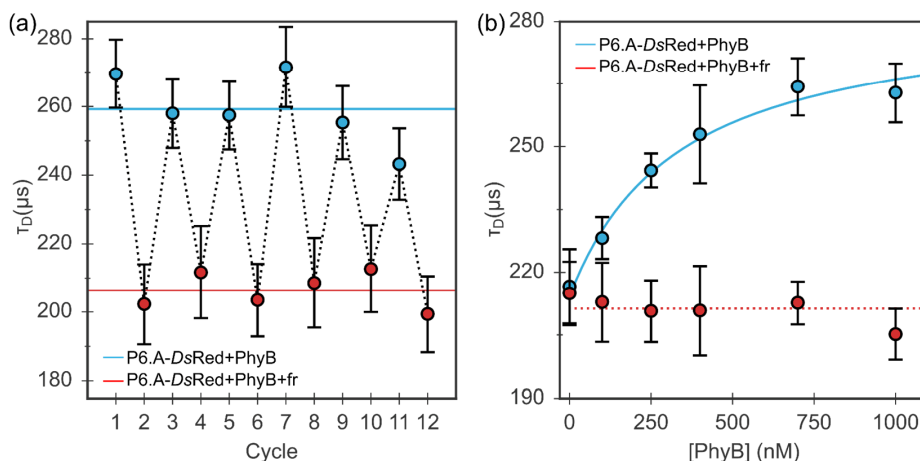


Figure 3: Fluorescence correlation spectroscopy (FCS) of the *PhyB*:PIF interaction (a) Reversible switching of the *AtPhyB*:P6.A-*DsRed* interaction. Odd cycle numbers (blue) correspond to measurements after red-light illumination, and even cycle numbers (red) are measurements with simultaneous far-red light illumination. The solid lines refer to the mean values of τ_D for the interacting and non-interacting states of (259 ± 9) μ s and (206 ± 5) μ s, respectively. (b) Correlation time τ_D of P6.A-*DsRed* as a function of *AtPhyB* PCM concentration under red light (blue) or far-red light (red). Data points correspond to the average of at least six independent measurements. The solid line shows a fit to a single-site binding isotherm (eq. (8)), and the dotted line represents the mean correlation time of (211 ± 3) μ s for the non-interacting state.

Resolving the Kinetics of the Light-Dependent Association Reaction

Having analyzed the binding equilibrium between P6.A-*DsRed* and *AtPhyB* PCM by concurrent fluorescence approaches, we next sought to resolve the kinetics of this interaction. To this end, we harnessed as a readout the resonance energy transfer from *DsRed* to the Pfr state of the *AtPhyB* PCM and the resultant decrease in fluorescence. To record association kinetics, we incubated a constant 20 nM P6.A-*DsRed* with between 1000 and 3000 nM *AtPhyB* PCM and illuminated with far-red light prior to the experiment. We then exposed the samples to red light and immediately started monitoring the system response over time by fluorescence at excitation and emission wavelengths of (540 ± 5) nm and (590 ± 5) nm (Fig. 4a and Suppl. Fig. S6a-d). To record dissociation kinetics, samples were illuminated with red light before the experiment and with far-red light at the onset of the measurement. At 1000 nM *AtPhyB* PCM, the association and dissociation reactions could be described by single-exponential functions with rate constants of (0.82 ± 0.03) s⁻¹ and (0.18 ± 0.01) s⁻¹, respectively. Control measurements by absorption spectroscopy (Suppl. Fig. S7) confirmed that at the intensities used, photoconversion by red and far-red light is not rate-limiting. Red light photoconverted *AtPhyB* from Pr to Pfr with a rate constant of (1.7 ± 0.1) s⁻¹, and far-red light drove

the conversion from Pfr to Pr with a velocity of $(0.60 \pm 0.01) \text{ s}^{-1}$. Moreover, although the available data are somewhat sparse and mainly pertain to phytochrome A, time-resolved measurements indicate that conformational changes within plant phytochromes ensuing the photochemical events are generally complete within milliseconds [46,47]. Taken together, the fluorescence time courses observed presently are hence mostly governed by the association and dissociation kinetics of the P6.A-DsRed:AtPhyB PCM complex. As the AtPhyB PCM concentration much exceeded that of P6.A-DsRed, we evaluated the data according to pseudo first-order kinetics, that is, single-exponential decays. A plot of the observable, unimolecular rate constant k_{obs} against the AtPhyB PCM concentration yields the bimolecular association and the unimolecular dissociation rate constants k_1 and k_{-1} as the slope and the ordinate intercept of the linear correlation (Fig. 4b). For the Pfr state of the AtPhyB PCM, we determined parameter values of $k_1 = (3.9 \pm 0.5) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = (0.42 \pm 0.06) \text{ s}^{-1}$. Correcting for the fact that red light populates the Pfr state only to around 56% extent rather than unity, k_1 amounted to $(7.0 \pm 0.9) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Based on the two rate constants, we calculate a K_d value of $(600 \pm 120) \text{ nM}$ for the Pfr state which is somewhat higher than the values determined by equilibrium measurements but nonetheless in reasonable agreement. For the Pr state, k_{obs} showed no pronounced dependence on AtPhyB PCM concentration, thus precluding a reliable determination of k_1 $[(0.8 \pm 5.0) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}]$ but allowing determination of $k_{-1} = (0.19 \pm 0.09) \text{ s}^{-1}$.

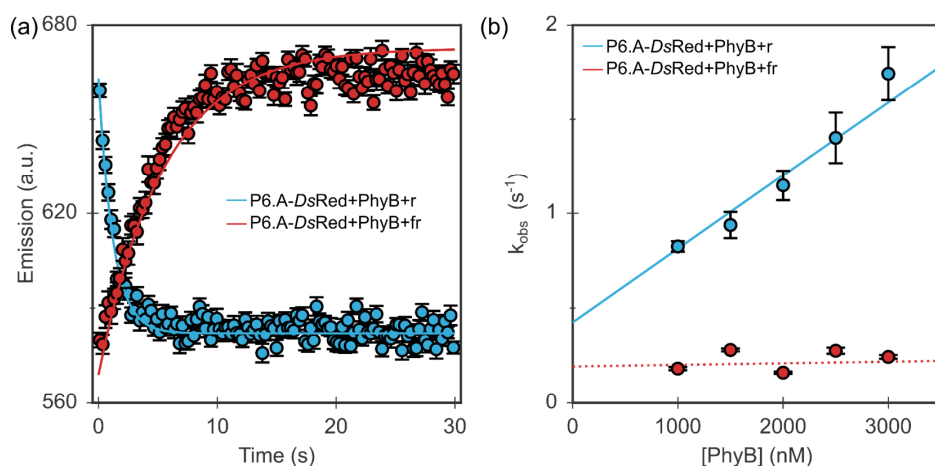


Figure 4: Association and dissociation kinetics of AtPhyB PCM and P6.A-DsRed. (a) Fluorescence of P6.A-DsRed in the presence of 1000 nM AtPhyB PCM was monitored over time upon exposure to red light (650 nm, blue symbols) or far-red light (720 nm, red symbols). Data points correspond to the average of three independent experiments and were fitted to eq. (2). (b) The observable rate constant k_{obs} for the association (blue) and dissociation reactions (red) under red and far-red light, respectively, as a function of AtPhyB concentration were fitted to a linear function (eq. (3)). Data points correspond to the average of three independent measurements.

Finally, we compared the experimentally determined association rate constant for the Pfr state to the bimolecular diffusional encounter rate constant expected for the P6.A-DsRed:AtPhyB PCM pair. To this end, we inserted the transversal diffusion coefficients D for the two proteins, as determined by FCS, and their hydrodynamic radii R_H , derived from the Stokes-Einstein relation

(Table 1), into the von Smoluchowski equation [48]. We thus calculated a diffusion-controlled bimolecular encounter rate constant of $(6.5 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, around 10 times faster than the experimentally observed association rate constant.

Discussion

Interactions between plant phytochromes and their interacting factors underpin intricate responses to red and far-red light in both nature and biotechnological processes. Despite the relevance of these interactions and their early identification more than two decades ago [4,16,17], their quantitative analysis has remained incomplete. Against this backdrop, we recently investigated the interactions between the PhyB PCM and the PIFs 3 and 6, all from *A. thaliana*, and provided quantitative data on the binding equilibria as a function of red and far-red light [32]. The identification of sequence determinants in the PIF proteins governing their interaction with AtPhyB informed the construction of light-regulated cellular circuits, some with enhanced performance. As amply demonstrated for certain UV and blue-light receptors [49], light-gated PPIs represent a versatile means of optogenetically regulating diverse cellular processes. To expand the application scope of the plant phytochrome system, we here characterized in depth the light-dependent interaction between a tetramerized AtPIF6 variant and the AtPhyB PCM. Multivalent, well-characterized and light-regulated PPIs stand to unlock additional physiological processes and parameters for optogenetic control, for example the clustering and concomitant activation of receptors or the dynamic formation and dissolution of protein-based subcellular compartments [50,51]. We thus investigated the light-dependent association equilibrium between the homotetrameric P6.A-DsRed and the AtPhyB PCM by an arsenal of fluorescence methods. Within error, the different methods consistently yielded dissociation constants for the Pfr state on the order of 200 nM, similar to the value of 280 nM observed earlier in a monomeric context [32]. We also demonstrated that the interaction between the two proteins can be repeatedly and bimodally toggled by red and far-red light. The analyses further revealed that within the homotetrameric P6.A-DsRed context, one P6.A entity binds AtPhyB PCM in a 1:1 stoichiometry. In addition, we did not observe any cooperativity of binding and hence conclude that the P6.A:AtPhyB PCM interaction is unperturbed by the linkage to DsRed. Strikingly, the FCS measurements showed little binding to the Pr state which contrasts with the present and the past fluorescence anisotropy experiments [32] that both exhibited a weak, micromolar interaction. We ascribe this discrepancy to the simultaneous illumination with far-red light during the FCS measurements, which rapidly returns AtPhyB PCM to its Pr state. By contrast, the anisotropy measurements were performed without far-red illumination, and hence minor Pfr fractions might be present and associate with the P6.A protein. As a corollary, the affinities determined by anisotropy after far-red light exposure should be considered lower estimates of the true dissociation constants in the Pr state.

We monitored the association and dissociation kinetics to more precisely analyze the phytochrome:PIF interaction and to assess the origin of its pronounced light dependence. Within the Pfr state, the AtPhyB PCM and P6.A-DsRed exhibited fast association with a bimolecular rate

constant of $7.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and moderately paced dissociation of about 0.4 s^{-1} . For the Pr state, the association was too slow to be reliably measured, and the dissociation was around half as fast as that in the Pfr state. Unexpectedly, the differential affinity of the Pr and Pfr states is therefore entirely rooted in the association reaction, rather than the dissociation. This finding is somewhat puzzling, given that tight molecular interactions among proteins and ligands are commonly achieved by very slow dissociation reactions [52]. We compared the observed bimolecular association of P6.A-DsRed and AtPhyB PCM in its Pfr state to the velocity of diffusional encounter between these two entities. Based on the transversal diffusion coefficients and the hydrodynamic radii of the two proteins, all derived from FCS measurements, an encounter rate constant of $6.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ results. This diffusion-controlled upper limit exceeds the experimentally observed association rate constant by only around 10-fold, thus testifying to the efficiency of the association reaction upon exposure to red light. Not least, this observation has direct implications for the engineering of plant phytochromes and their optogenetic application: there is little scope in further enhancing PIF affinity through optimization of the association reaction; if the affinity is to be improved, it will be through slowing down the dissociation reaction. We note that more extended PIF6 variants, comprising the APB.B segment in addition to APB.A, indeed exhibit stronger binding to AtPhyB PCM than P6.A [32]. Based on the current study, we suggest that this higher affinity mainly originates from a slower dissociation reaction.

Taken together, we provide novel insights into the equilibrium and kinetics of the plant phytochrome:PIF interaction. The hitherto unknown, quantitative parameters underlying this interaction now stand to inform the analysis of phytochrome signaling in plants and pertinent applications in optogenetics and biotechnology. More generally, the multimodal fluorescence approach we implemented currently is well suited for the analysis of other light-dependent interactions, including in other well-known and widely used photoreceptors like plant cryptochromes [53] or certain light-oxygen-voltage proteins [54,55].

Material & Methods

Molecular biology and protein expression

The P6.A-DsRed construct was generated in a pET-19b vector background (Novagen, Merck, Darmstadt, Germany) by Gibson assembly [56] of gene fragments PCR-amplified from plasmids encoding P6.A (residues 14-53 of *A. thaliana* PIF6) [32] and DsRed-Express 2 [37], respectively. A linker of the sequence DSAGSAGSAG was thereby inserted between the P6.A and DsRed moieties. Within the resultant plasmid pLH037, the protein is fused to an N-terminal hexahistidine SUMO tag [57], and expression is under the control of a T7-lacO promoter. Protein expression was carried out in *Escherichia coli* LOBSTR cells [58], and purification was done by immobilized metal ion affinity chromatography, as previously established for AtPIF-EYFP variants [32]. For production of the *A. thaliana* PhyB PCM (residues 1-651), the plasmid pDG282 [32] was used that additionally encodes

the *Synechocystis* sp. heme oxygenase 1 and *pcyA* for production of the phycocyanobilin chromophore [59]. Protein expression and purification were performed as before [32].

The purified proteins were analyzed at 22°C by absorption spectroscopy on an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany). Concentrations of P6.A-*DsRed* and *AtPhyB* PCM were determined using extinction coefficients of 35600 M⁻¹ cm⁻¹ at 554 nm [37] and of 47600 M⁻¹ cm⁻¹ at 672 nm [32], respectively. Absorption spectra of the *AtPhyB* PCM were recorded prior to and after illumination with light-emitting diodes with emission maxima of (650 ± 15) nm (intensity 5.6 μW cm⁻²) and (720 ± 15) nm (0.7 μW cm⁻²), respectively. The Pr/Pfr ratio of 0.44/0.56 upon 650-nm illumination and the spectrum of the pure Pfr state were calculated as described in [60].

Fluorescence intensity measurements

Fluorescence analyses were carried out on a Jasco FP-6500 spectrofluorimeter (Jasco, Pfungstadt, Germany). All measurements were performed in buffer A (20 mM HEPES/HCl pH 7.3, 10 mM NaCl, and 100 μg mL⁻¹ bovine serum albumin). Fluorescence emission spectra of P6.A-*DsRed* were recorded at concentrations of 20, 30 and 40 nM at an excitation wavelength of (540 ± 5) nm in the presence of 2000 nM *AtPhyB* PCM following illumination with red (650 nm) or far-red (720 nm) light. The fluorescence quantum yield Φ_F of P6.A-*DsRed* in the presence of the non-interacting Pr state of the *AtPhyB* PCM was assumed to equal 0.42, corresponding to that of the isolated *DsRed*-Express 2 [37]. To determine Φ_F of P6.A-*DsRed* when in complex with the Pfr state of the *AtPhyB* PCM (i.e. after red-light exposure), the emission integral was plotted against the P6.A-*DsRed* concentration according to [61]. Characteristic distances R_0 for resonance energy transfer from P6.A-*DsRed* to the Pfr state of the *AtPhyB* PCM were calculated on the basis of the relevant emission and absorption spectra [39].

To monitor the binding equilibrium, 20 nM P6.A-*DsRed* was incubated at 22°C with *AtPhyB* PCM concentrations between 1000 nM and 3000 nM, and fluorescence was recorded at excitation and emission wavelengths of (540 ± 5) nm and (590 ± 5) nm, respectively. During the measurement, the samples were illuminated with red light (650 nm) to partially populate the Pfr state of the *AtPhyB* PCM, or far-red light (720 nm) to drive the conversion to Pr. The ratio $\tilde{F}$ of fluorescence intensities obtained for red and far-red light conditions was plotted against the *AtPhyB* PCM concentration and fitted to a single-site binding isotherm (eq. (1)).

$$\tilde{F} = \tilde{F}_0 + (\tilde{F}_1 - \tilde{F}_0) \times [\text{AtPhyB}] / ([\text{AtPhyB}] + K_d) \quad (1)$$

where $\tilde{F}_0$ and $\tilde{F}_1$ are the fluorescence intensity ratios of free and fully bound P6.A-*DsRed*, respectively, K_d is the dissociation constant, and $[\text{AtPhyB}]$ is the concentration of the *AtPhyB* PCM. Unless stated otherwise, data fitting was done using Fit-o-mat version 0.781 [62]. To record association kinetics, the samples were illuminated with far-red light prior to the measurement. At the start of the measurement, the samples were exposed to red light, and fluorescence was recorded over time in 10-ms intervals. To record dissociation kinetics, the samples were first

illuminated with red light and then exposed to far-red light at the start of the time course. The resultant pseudo first-order kinetics were fitted to eq. (2):

$$F(t) = F_0 + (F_1 - F_0) \times \exp(-k_{\text{obs}} \times t) \quad (2)$$

where F_0 and F_1 are the fluorescence intensities at $t \rightarrow \infty$ and $t=0$, respectively, and k_{obs} the observable rate constant. The values for k_{obs} were evaluated according to eq. (3) to determine the bimolecular rate constants k_1 for association of the P6.A-*DsRed*:*AtPhyB* PCM complex and the unimolecular rate constants k_{-1} for dissociation.

$$k_{\text{obs}} = k_1 \times [\text{AtPhyB}] + k_{-1} \quad (3)$$

where $[\text{AtPhyB}]$ is the concentration of the *AtPhyB* PCM.

Fluorescence anisotropy measurements

Using an Olis DSM 172 spectrophotometer (On-Line Instrument Systems, Bogart, USA), the fluorescence anisotropy of 40 nM P6.A-*DsRed* in buffer A at excitation and emission wavelengths of (540 ± 20) nm and (590 ± 20) nm was determined to be 0.282. All subsequent fluorescence anisotropy measurements were conducted on a CLARIOstar multimode microplate reader (BMG Labtech, Ortenberg, Germany). Fluorescence was recorded at excitation and emission wavelengths of (540 ± 20) nm and (590 ± 20) nm using a 566-nm long-pass dichroic mirror. The gain factors for the vertical and horizontal detection channels were adjusted such that the measured fluorescence anisotropy for P6.A-*DsRed* amounted to 0.282. To record binding isotherms, a constant 40 nM P6.A-*DsRed* was incubated with between 0 and 2000 nM *AtPhyB* PCM in black 384-well microtiter plates (Brand, Wertheim, Germany). Samples were illuminated for 2 min either with (640 ± 15) nm ($64 \mu\text{W cm}^{-2}$) or (750 ± 15) nm ($420 \mu\text{W cm}^{-2}$) light. Fluorescence anisotropy r was recorded immediately afterwards and evaluated according to eq. (4) which takes into account that the P6.A-*DsRed* fluorescence is slightly quenched when in complex with *AtPhyB* PCM [63].

$$r = \frac{r_0 + (R \times r_1 - r_0) \times f_b}{1 + (R - 1) \times f_b} \quad (4)$$

In eq. (4), r_0 and r_1 are the anisotropy values of P6.A-*DsRed* in its free form and when fully bound by the *AtPhyB* PCM, respectively, $R = 0.86$ is the fold change in P6.A-*DsRed* fluorescence quantum yield upon *AtPhyB* PCM binding, and f_b represents the fraction of bound P6.A-*DsRed*, which in turn is calculated according to eq. (5):

$$f_b = \frac{1}{2} \times \left\{ 1 + ([\text{AtPhyB}] + K_D)/c_{\text{tot}} - \sqrt{(1 + ([\text{AtPhyB}] + K_D)/c_{\text{tot}})^2/4 - [\text{AtPhyB}]/c_{\text{tot}}} \right\} \quad (5)$$

where $[\text{AtPhyB}]$ is the concentration of the *AtPhyB* PCM in the Pr or Pfr state, respectively, $c_{\text{tot}} = 40$ nM the concentration of P6.A-*DsRed*, and K_D the dissociation constant.

To determine the binding stoichiometry, an isotherm was recorded in the regime of stoichiometric binding at 700 nM P6.A-*DsRed*, i.e. well above the K_D value for the Pfr state. The *AtPhyB* PCM was illuminated with red light, added at between 0 and 5000 nM final concentration,

and anisotropy was recorded. The data points at low [AtPhyB, Pfr]/[P6.A-DsRed] ratios were fitted to a linear function where the slope corresponds to the binding stoichiometry in this regime.

Fluorescence correlation spectroscopy

The binding interaction between P6.A-DsRed and the AtPhyB PCM was probed by FCS measurements on a home-built epi-fluorescence confocal microscope. The setup was equipped with a high numerical aperture water-immersion objective (UPLSAPO 60x NA 1.2, Olympus) and a single-photon detector (PD050C0C, Micro-Photon-Devices), placed behind a pinhole with a diameter of 50 μm . The count rate of the avalanche diode was monitored with a digital I/O module (PCI-6221, National Instruments). A 25 μL droplet of the sample was excited at 510 nm from a pulsed diode laser (BDS-SM 510, Becker & Hickl GmbH) running at 20 MHz frequency. Fluorescence emission was detected through a combination of a long-pass (BLP01-514R Edge Basic, AHF Analysentechnik) and two short-pass filters (633 SP Edge Basic, AHF Analysentechnik), creating a 526 – 622 nm band-pass. For illuminating the sample with far-red light during the measurement, a high-power LED (720 nm) was mounted about 20 mm above the sample holder. The intensity of the LED light, measured at the sample holder, was about 12 mW cm^{-2} . In order to prevent evaporation of the sample during the measurement, the droplet was covered with a transparent plastic case. All measurements were done at 20°C.

For each sample, fluorescence decay and the autocorrelation were measured over 300 seconds in triplicate with a TCSPC module (SPC-130, Becker & Hickl GmbH). After-pulsing effects of the photon detector on the autocorrelation function were taken into account by correcting the recorded data according to eq. (6) [64]:

$$G(\tau) = G'(\tau) - \langle i_{sc} \rangle / \langle i \rangle \times (G_{sc}(\tau) - 1) \quad (6)$$

where τ is the lag-time, $G'(\tau)$ is the measured autocorrelation of the sample, and $G_{sc}(\tau)$ is the after-pulsing function of the detector. $G_{sc}(\tau)$ was recorded in a previous experiment with the uncorrelated emission of an LED torch light. The values $\langle i \rangle$ and $\langle i_{sc} \rangle$ are the mean count rates of the actual experiment and the measurement of $G_{sc}(\tau)$, respectively. The corrected autocorrelation was then fitted to the standard three-dimensional diffusion model:

$$G(\tau) = G_0 \times (1 + \tau/\tau_D)^{-1} \times (1 + \tau/(\gamma^2 \tau_D))^{-1/2} \quad (7)$$

where τ_D is the correlation time and γ is the axial ratio of the detection volume. The correlation amplitude $G_0 = 1/(\langle N \rangle \chi^2)$ includes the mean number of molecules $\langle N \rangle$ in the detection volume and a correction factor $\chi^2 = (\langle F \rangle / (\langle F \rangle - \langle b \rangle))^2$, where $\langle F \rangle$ and $\langle b \rangle$ are the count rates of the sample and a buffer blank, respectively. The buffer count rates were measured in the absence of P6.A-DsRed for each AtPhyB PCM concentration studied. The γ value was determined according to [65] with a Rhodamine 110 solution of known concentration [66], giving a value of $\gamma = 9$. The lateral radius ω_r of the detection volume for this setup was about 240 nm. Measurements were performed at a laser intensity of 2.0 KW cm^{-2} in the confocal volume. The correction for self-correlation and

fitting were done with a custom Python script. The resultant correlation times τ_D were plotted against the *AtPhyB* PCM concentration and fitted to a single-site binding isotherm (eq. (8))

$$\tau_D = \tau_0 + (\tau_1 - \tau_0) \times f_b \quad (8)$$

where τ_0 and τ_1 are the correlation times of free and fully bound P6.A-DsRed, respectively, and f_b is calculated according to eq. (5) with $c_{\text{tot}} = 20$ nM as the constant concentration of P6.A-DsRed.

To study interactions of the *AtPhyB* PCM in its Pfr state, samples were illuminated with red light (650 nm) for 1 min prior to the measurement, followed by determination of τ_D . To access the Pr state, measurements were conducted under simultaneous illumination with far-red light (720 nm). The influence of the 510-nm pulsed diode laser on the interaction was assessed by comparing measurements in the presence and absence of 720-nm simultaneous illumination.

Fluorescence lifetimes were determined by tail-fitting the fluorescence decay curves to the sum of two exponential functions (eq. (9)):

$$F(t) = A_0 + A_1 \times \exp(-t/\tau_1) + A_2 \times \exp(-t/\tau_2) \quad (9)$$

where τ_1 and τ_2 are the fluorescence lifetime components, A_1 and A_2 are the corresponding amplitudes, and A_0 is the background. Fitting was performed with the FluoFit software (version 4.6.6.0, PicoQuant GmbH) using the global fitting routine. The slower fluorescence lifetime component τ_2 was plotted against the *AtPhyB* PCM concentration and fitted to a single-site binding isotherm (eq. (10))

$$\tau_2 = \tau_f + (\tau_b - \tau_f) \times f_b \quad (10)$$

where τ_f and τ_b are the slow fluorescence lifetime components of free and fully bound P6.A-DsRed, respectively, and f_b is calculated according to eq. (5) with $c_{\text{tot}} = 20$ nM as the constant concentration of P6.A-DsRed.

The transversal diffusion of the *AtPhyB* PCM was examined by FCS on a commercial time-resolved confocal fluorescence microscope (MicroTime 200, PicoQuant GmbH). A sample droplet of 25 μL was excited with a wavelength of 639 nm from a 20 MHz pulsed diode laser (LDH-D-C 640, PicoQuant GmbH, pulse width ≤ 500 ps), and the emission was collected with a high numerical aperture water-immersion objective (UPLSAPO 60x NA 1.2, Olympus). The fluorescence was detected with a single-photon avalanche diode (SPAD Excelitas Technologies Corp) through a 650-720 nm band-pass filter and a pinhole with a diameter of 100 μm . The microscope was calibrated with an Atto-655 dye solution (ATTO-TEC GmbH) [66], yielding a lateral radius ω_r of 350 nm of the detection volume. Measurements were done at an intensity of 1.3 kW cm⁻² within the confocal volume. The autocorrelation was analyzed with the SymPhoTime 64 software package (PicoQuant GmbH) which automatically corrected the data for after-pulsing effects. All measurements were done at a temperature of 20°C.

Hydrodynamic radii R_H were calculated according to the Stokes-Einstein equation (11):

$$R_H = k_b T / (6\pi\eta D) \quad (11)$$

where k_b is the Boltzmann constant, T the absolute temperature, η the viscosity (taken to be 1 mPa s at 20°C), and D the transversal diffusion coefficients, as determined by FCS. We next calculated the diffusional encounter rate constant k_{bi} from the hydrodynamic radii R_H and the diffusion coefficients D via the von Smoluchowski equation (12):

$$k_{bi} = 4\pi N_A \times \sum R_H \times \sum D \quad (12)$$

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Supplementary Information

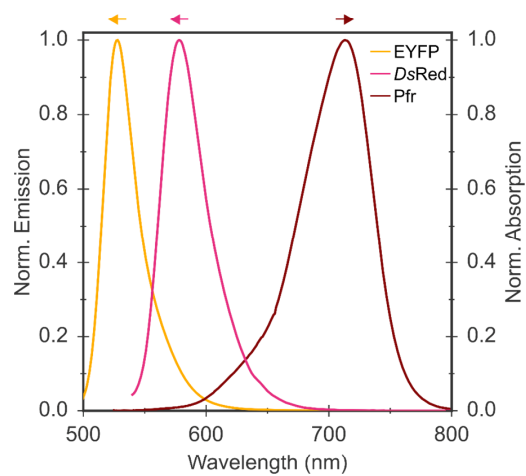


Figure S1: Normalized Pfr-state absorption of the AtPhyB PCM (brown) and the normalized emission of EYFP (yellow) and DsRed (magenta).

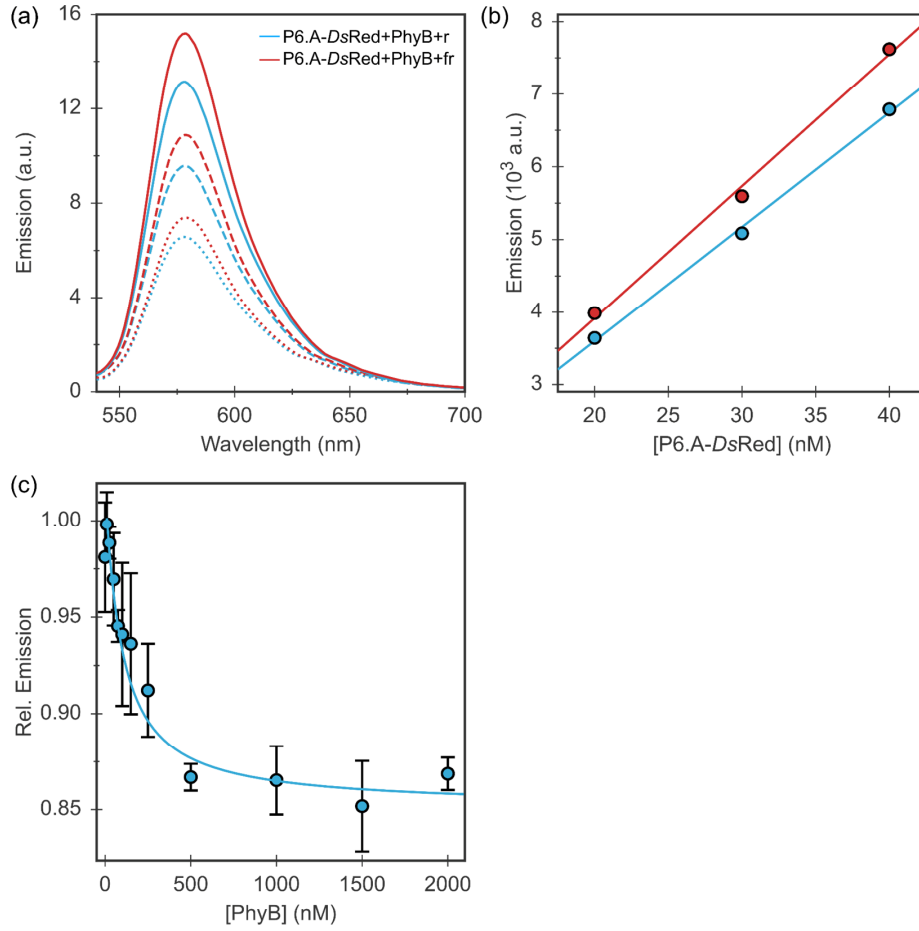


Figure S2: Analysis of the P6.A-DsRed:AtPhyB PCM interaction by fluorescence intensity measurements. (a) Fluorescence emission of P6.A-DsRed in the presence of 2000 nM AtPhyB PCM after exposure to far-red light (720 nm, red line) or red light (650 nm, blue line). Full, dotted and dashed lines represent P6.A-DsRed concentrations of 20, 30 and 40 nM. (b) Integrated fluorescence emission as a function of P6.A-DsRed concentration. Data points correspond to three independent measurements and were fitted by linear regression. (c) Normalized fluorescence intensity of P6.A-DsRed as a function of AtPhyB PCM concentration (see main text). Data points correspond to three independent measurements and were fitted to a single-site isotherm (eq. (1)).

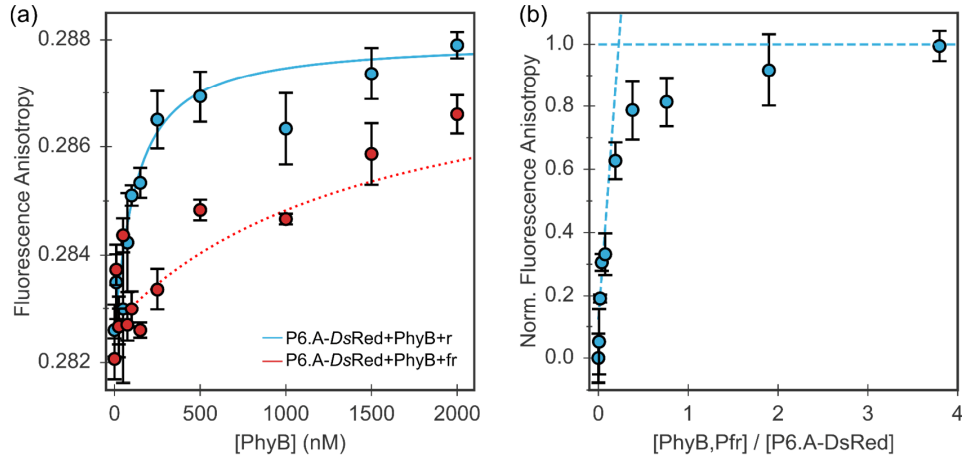


Figure S3: Analysis of the P6.A-DsRed:AtPhyB PCM interaction by fluorescence anisotropy measurements. (a) Fluorescence anisotropy of P6.A-DsRed as a function of AtPhyB PCM concentration after exposure to far-red light (720 nm, red line) or red light (650 nm, blue line). Data points correspond to three independent measurements and were fitted to single-site binding models (eq. (4)). (b) Fluorescence anisotropy measured after red-light exposure in the stoichiometric regime at 700 nM P6.A-DsRed. Normalized anisotropy as a function of the molar ratio of AtPhyB PCM over P6.A-DsRed. The dashed blue lines represent the maximum value of 1.0 and a linear fit to the data at low ratios, respectively.

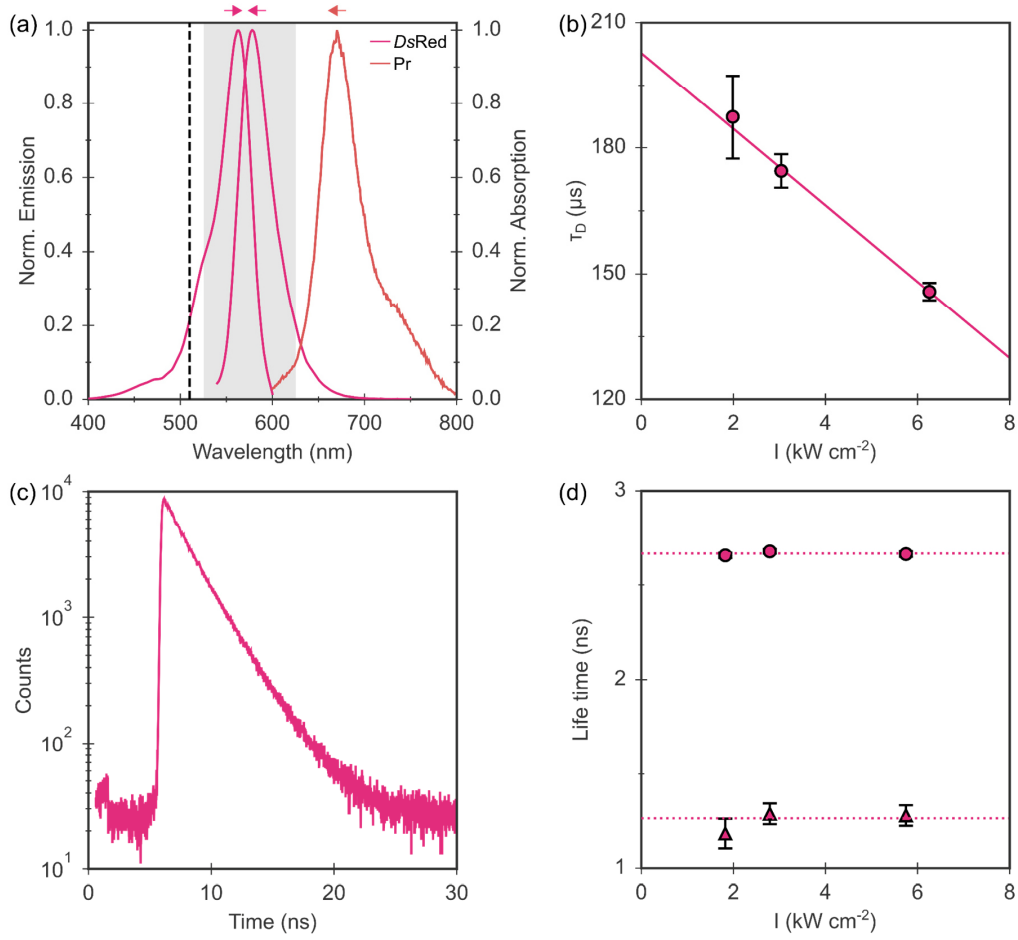


Figure S4: Fluorescence correlation spectroscopy (FCS). (a) Fluorescence excitation and emission spectra of *DsRed* (magenta) and emission spectrum of the *AtPhyB* PCM in its *Pr* state (red). The dashed line denotes the wavelength 510 nm of the laser used in the FCS experiments. Fluorescence emission was detected between 520 nm and 640 nm (gray shading). (b) Correlation time τ_D of the *DsRed* fluorophore as a function of the laser intensity. (c) Fluorescence decay for *DsRed* at an excitation laser intensity of 2.0 kW cm^{-2} . (d) Fluorescence lifetimes of *DsRed* as a function of laser intensity. A two-component model was used to fit the fluorescence decay data (eq. (9)), yielding fluorescence lifetimes of $(1.19 \pm 0.04) \text{ ns}$ and $(2.64 \pm 0.02) \text{ ns}$, respectively (circles and triangles). Data points correspond to three independent measurements.

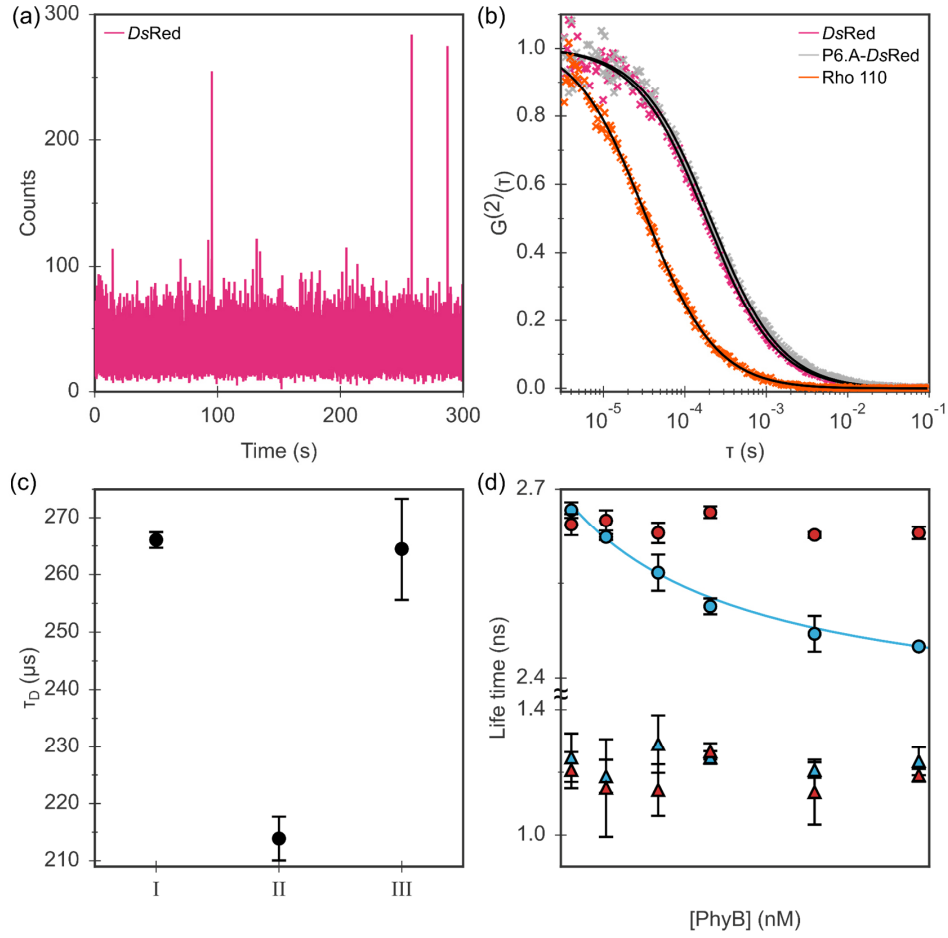


Figure S5: Analysis of the P6.A-DsRed:AtPhyB PCM interaction by fluorescence correlation spectroscopy (FCS). (a) Fluorescence signal of DsRed excited with a 510-nm laser at an intensity of 2.0 kW cm^{-2} . (b) Normalized autocorrelation functions for Rhodamine 110 (Rho 110, orange), DsRed (magenta) and P6.A-DsRed (gray), with black curves representing fits to a standard three-dimensional diffusion model (eq. (7)). (c) Correlation time τ_D for P6.A-DsRed in the presence of 700 nM AtPhyB PCM. (I) after red illumination (650 nm), (II) under simultaneous far-red illumination (720 nm) throughout the experiment, (III) after far-red illumination prior to the experiment. (d) Fluorescence lifetimes of P6.A-DsRed as a function of increasing concentration of AtPhyB PCM under far-red light (blue symbols) or red light (red symbols). A two-component model was used to fit the fluorescence decay data (eq. (9)), yielding two fluorescence lifetimes, depicted as circles and triangles. Data points correspond to three independent measurements, and those for the slower phase were fitted to a single-site isotherm (eq. (10)).

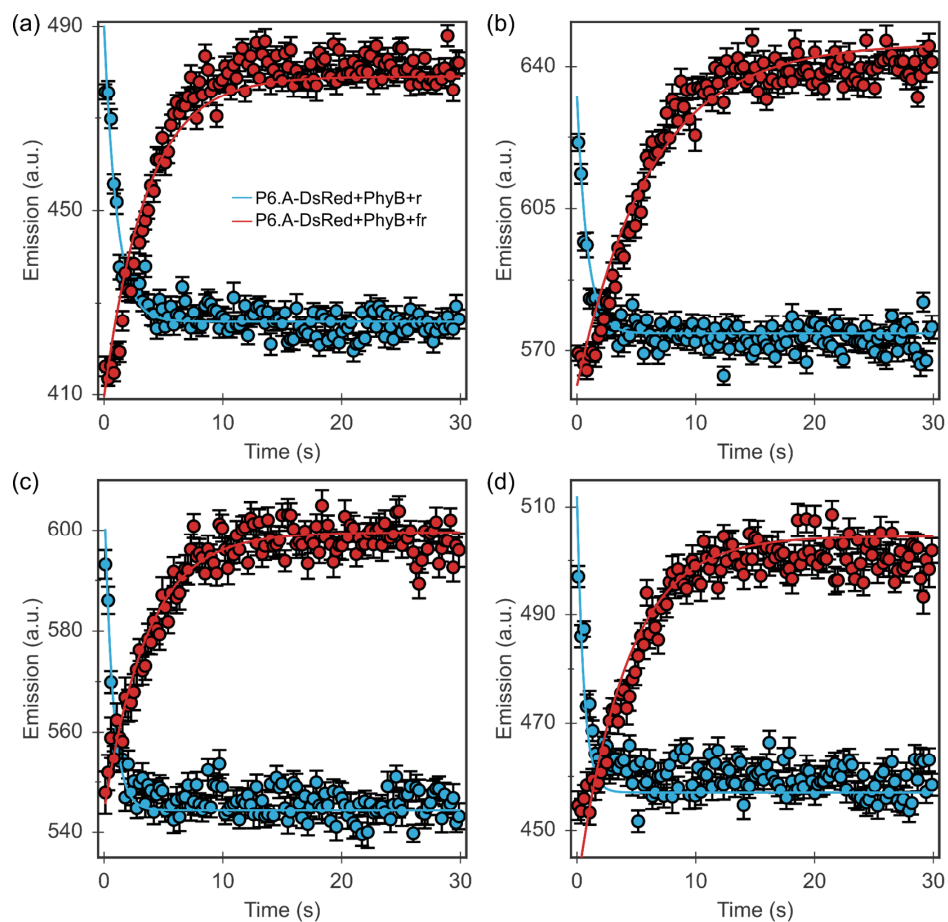


Figure S6: Association and dissociation kinetics of AtPhyB PCM and P6.A-DsRed. Fluorescence of P6.A-DsRed in the presence of AtPhyB PCM over time upon exposure to red light (650 nm, blue symbols) or far-red light (720 nm, red symbols). Data points correspond to the average of three independent experiments and were fitted to eq. (2). Data were acquired for AtPhyB PCM concentrations of 1.5 (a), 2.0 (b), 2.5 (c) and 3.0 μM (d).

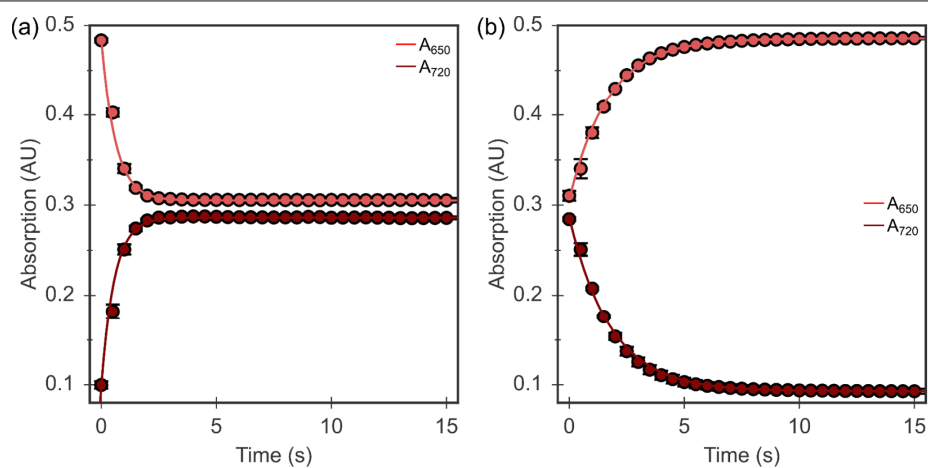


Figure S7: Photoconversion kinetics of AtPhyB PCM under exposure to red (650 nm) and far-red light (720 nm), using the conditions of the experiments in Fig. 3 and Suppl. Fig. S6. (a) Absorption of the AtPhyB PCM at 650 nm (red symbols) and 720 nm (brown symbols) upon illumination with red light (650 nm). (b) Absorption of the AtPhyB PCM at 650 nm (red) and 720 nm (brown) upon illumination with far-red light (720 nm).

5.3 Manuskript III

Titel: „*Arabidopsis thaliana* phytochrome B incorporates biliverdin and undergoes light-dependent interactions”

Autoren: Golonka, D.; Kuznetsova, V.; Ihalainen, J. A.; Möglich, A.

Noch nicht eingereicht

Arabidopsis thaliana Phytochrome B Incorporates Biliverdin and Undergoes Light-dependent Interactions

David Golonka^{1,†}, Valentyna Kuznetsova, Janne A. Ihalainen, Andreas Möglich^{1,2,3,*},[†]

¹Lehrstuhl für Biochemie, Photobiochemie, Universität Bayreuth, 95447 Bayreuth, Germany.

²Bayreuth Center for Biochemistry & Molecular Biology, Universität Bayreuth, 95447 Bayreuth, Germany.

³North-Bavarian NMR Center, Universität Bayreuth, 95447 Bayreuth, Germany.

*to whom correspondence may be addressed: andreas.moeglich@uni-bayreuth.de

[†]ORCID identifiers: D.G. 0000-0003-4101-4591; V.K. xxxx-xxxx-xxxx-xxxx; J.I. xxxx-xxxx-xxxx-xxxx; A.M. 0000-0002-7382-2772

Abstract

Plant phytochromes monitor the ratio of red to far-red light via the plant specific chromophore phytochromobilin, a precursor of biliverdin (BV) utilized by bacteriophytochromes. Herein, we show that the photosensory core module of *Arabidopsis thaliana* phytochrome B (AtPhyB-PCM) is in fact able to covalently bind BV and is still able to interact with its partner protein PIF3. Spectral analysis of this biliverdin-bound AtPhyB-PCM reveals more similarity to the PCB-bound AtPhyB than to bacterial phytochromes with BV. We propose a new mechanism for covalent attachment of BV in AtPhyB which explains the low yield of BV incorporation previously reported. Furthermore, we optimized AtPhyB by introducing a histidine at position 578 yielding BVB-PhyB that boosted assembly rate with BV to 87 % during co-expression of BVB-PhyB with *Synechocystis sp.* heme oxygenase 1 without external BV addition. BVB-PhyB stands to improve the application of the AtPhyB-PIF interaction systems for optogenetics and biotechnology tremendously.

Keywords

chromophore; optogenetics; phytochrome; protein-protein interaction; sensory photoreceptor; signal transduction; tetrapyrrole

Introduction

Light is an essential environmental cue for many organisms. Sensory photoreceptors are proteins that are able to sense light conditions and thereby, alter biological response (Hegemann 2008; Möglich et al. 2010). The spectral sensitivity of photoreceptors depends on the respective receptor family and ranges the whole spectra of light from UV to far-red (Ziegler und Möglich 2015). Phytochromes (Phy) are a class of photoreceptors responsible for monitoring red and far-red light conditions and were identified in numerous organisms such as bacteria, fungi, cyanobacteria, algae

and plants (Rockwell et al. 2006). While Phys mediate phototaxis in bacteria (Fiedler et al. 2005), their role in plants spans the whole development process from seedling germination over shade avoidance to flowering (Bae und Choi 2008; Hughes 2013; Pham et al. 2018; Rockwell und Lagarias 2020).

The architecture of Phys generally consists of a N-terminal photosensory core module (PCM), which is necessary for light sensing, and a C-terminal output module (OPM) that mediates biological response and therefore, serves as effector (Fig. 1a). One PAS (Per-ARNT-Sim) (Möglich et al. 2009), a GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhIA) (Aravind und Ponting 1997) and the PHY (phytochrome-specific) domain (Essen et al. 2008; Rockwell und Lagarias 2010; Yang et al. 2008) form the PCM with the linear tetrapyrrole (bilin) chromophore for light sensing embedded into the GAF domain. In addition, plant phytochromes utilize a N-terminal extension (NTE) which precedes the PAS domain of the PCM (Horsten et al. 2016). The type of bilin chromophore nestled within the GAF domain mainly dependence on the organism of origin. Biliverdin (BV), a product of oxidative degradation of heme by heme oxygenase (FRANKENBERG und Lagarias 2000), is widely employed in bacterial phytochromes (Bhoo et al. 2001; Kaberniuk et al. 2016; Lamparter et al. 2003; Shcherbakova et al. 2015b; Wagner et al. 2008). Plants and cyanobacteria metabolize BV into phytochromobilin (PΦB) or phycocyanobilin (PCB), respectively (Rockwell et al. 2006; Rockwell und Lagarias 2010). While plant phytochromes can be utilized using PCB (Golonka et al. 2019; Müller et al. 2013; Toettcher et al. 2013), an substitution of PΦB by BV appears unlikely as a result of the distinct binding mechanisms associated with the respective chromophores (Fig. 1b). In bacterial Phys, BV is covalently attached to a cysteine in the PAS domain via the C3² atom (Karniol et al. 2005; Lamparter et al. 2004; Takala et al. 2014a; Wagner et al. 2007), whereas plant and cyanobacterial Phys bind their respective chromophore at the C3¹ position via a cysteine inside the GAF domain (Burgie et al. 2014; Essen et al. 2008). Independent of their binding mechanism, bilins in plant and bacterial phytochromes undergo a Z to E isomerization between the double bond C15=C16 upon red light illumination (Fig. 1c). Photon absorption of the 15Z state (Pr) triggers the formation of short-lived Lumi-R and Meta-R intermediates before the (meta)stable 15E state (Pfr) is reached (Fig. 1d) (Ihalainen et al. 2018; Rockwell und Lagarias 2010). The Pfr state returns to the Pr state either in a slow thermal reversion or via absorption of far-red light which involves the formation of Lumi-F and Meta-F states (Chen et al. 1997). The number of intermediates during Pr ↔ Pfr conversion as well as their stability varies depending on the type of phytochrome (Björling et al. 2016; Borucki 2006; Kübel et al. 2020; Wagner et al. 2008). A protrusion of the PHY domain called tongue region, which closely interacts with the chromophore binding pocket in the GAF domain, undergoes structural refolding during the Pr ↔ Pfr transition resembled by a β-hairpin to α-helix conversion and vice versa (Anders et al. 2013; Burgie et al. 2016; Takala et al. 2014a). Furthermore, larger-scale structural changes occur inside the PCM which are propagated downstream to the OPM resulting in biological activity.

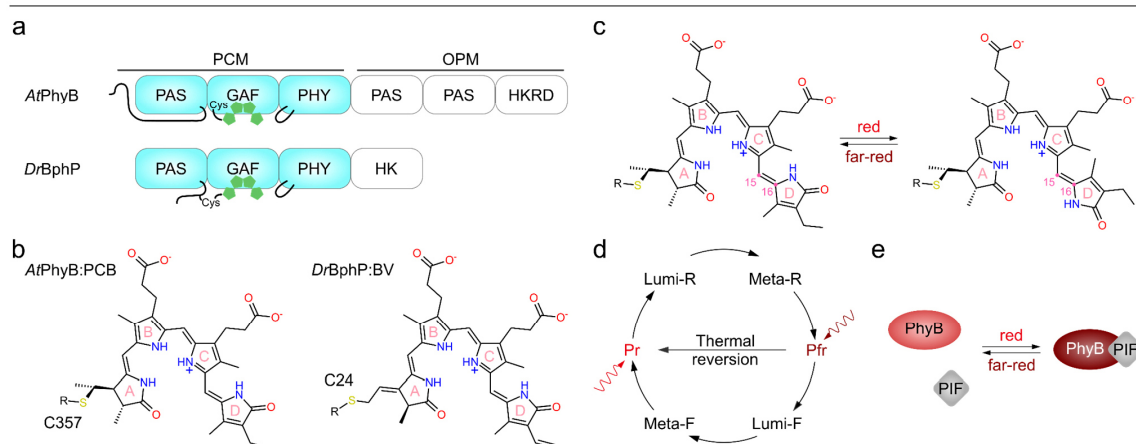


Figure 1: Key characteristics of Phytochromes. (a) Domain architecture of *Arabidopsis thaliana* phytochrome B (AtPhyB) and *Deinococcus radiodurans* bacteriophytochrome (DrBphP). The photosensory core module (PCM) consist of PAS, GAF and PHY domains, while the output module (OPM) is a histidine-kinase-related domain (HKRD) in AtPhyB and a histidine kinase (HK) in DrBphP. (b) Difference in chromophore binding between plant and bacterial phytochromes. (c) Red and far-red light mediated photoisomerization between the C15 and the C16 atoms of bilin chromophores in phytochromes through the example of PCB. (d) Red light absorption triggers the photocycle of phytochromes which reverts by thermal reversion or after absorption of far-red light. (e) Light-dependent interaction between AtPhyB and phytochrome interacting factors (PIF).

The OPM of bacterial phytochromes, which generally consists of a histidine kinase, serves as effector that phosphorylates respective response regulators (Bhoo et al. 2001; Quail 1997; Rockwell und Lagarias 2010). However, plant Phys do not act as histidine kinases as the conserved histidine autophosphorylation site is missing (Yeh und Lagarias 1998). Contrary to other phytochromes, plant Phys possess two PAS domains, PAS-A and PAS-B, followed by a histidine-kinase related domain (HKRD) with reported serine/threonine kinase activity (Yeh und Lagarias 1998). Furthermore, plant phytochromes, most prominently the phytochromes A-E from *Arabidopsis thaliana* (AtPhyA-E), are known to undergo light-dependent protein:protein interactions with partner proteins, foremost phytochrome interacting factors (PIFs), even in the absence of the OPM (Fig. 1e) (Bae und Choi 2008; Golonka et al. 2019; Golonka et al. 2020; Khanna et al. 2004; Matsushita et al. 2003; Ni et al. 1998; Pham et al. 2018, 2018; Shimizu-Sato et al. 2002). PIFs are transcription factors regulating gene expression as homodimer and heterodimer or via interaction with numerous other interacting factors (Pham et al. 2018). Beyond its biological relevance, the light controlled AtPhyB:PIF interaction system has found countless applications in the field of synthetic biology to control and alter cellular behavior (Levskeya et al. 2009; Müller et al. 2013; Shimizu-Sato et al. 2002; Toettcher et al. 2013), also known as optogenetics (Deisseroth et al. 2006). Intriguingly, only the first 100 amino acids which encode a so called active phytochrome B binding (ABP) motif are necessary for interaction with AtPhyB (Khanna et al. 2004). Recently, we were able to quantify the interaction of AtPhyB and PIFs (Golonka et al. 2019; Golonka et al. 2020), reduced the ABP motif even further in size (Golonka et al. 2019), and showed that the interaction is encoded in the association kinetic of AtPhyB and PIFs (Golonka et al. 2020). Light-dependent protein:protein interaction systems possess significant advantages compared to constitutive or chemically inducible dimerization systems such as spatial and temporal precision; reversibility and robust switching between active and inactive

states (Golonka et al. 2020). However, addition of external chromophore is needed when applying the system in mammalian cell culture or higher organisms which limits application. A bacterial phytochrome-based protein:protein interaction system, recently published (Kaberniuk et al. 2016; Redchuk et al. 2017), stands to overcome this disadvantage, but quantitative information of the systems capabilities are largely missing. Therefore, a plant phytochrome with the ability of binding BV while still retaining red/far-red light dependent interaction with PIFs would stand to revolutionize the applicability of the AtPhyB:PIF system.

Herein, we show that AtPhyB(1-650) (AtPhyB-PCM) contrary to previous reports (Li und Lagarias 1992; Rockwell und Lagarias 2010), is in fact able to bind BV without further modification, albeit to a lesser extent. Introduction of the mutation R578H into AtPhyB-PCM improved assembly rate with BV to 87 % without external chromophore addition. The resulting biliverdin bound AtPhyB-PCM R578H (BVB-PhyB) still interacts with its partner PIF3 with only a minor loss in affinity. Spectral comparison of AtPhyB-PCM with PCB or BV and the *Deinococcus radiodurans* bacterial phytochrome (DrBphP-PCM) with BV reveals striking similarities between AtPhyB-PCM with BV and PCB which we attribute to alternative binding mechanism for BV in plant phytochromes compared to bacterial phytochromes.

Results

Arabidopsis thaliana phytochrome B incorporates biliverdin

While previous reports state that AtPhyB can bind BV only poorly or not all (Rockwell und Lagarias 2010; Spudich und Briggs 2005) we challenged this hypothesis by co-expressing AtPhyB-PCM with *Synechocystis* sp. heme oxygenase 1 (HO1) in *E. coli* cells for 16 h and supplementation of excessive amounts of BV during purification. To our surprise, the resulting AtPhyB-PCM did in fact bind BV yielding an assembly rate of about 62 % (Table 1). As a result, the question arose whether AtPhyB-PCM with BV could be produced only with endogenous BV. Under standard expression conditions of 16 h at 18°C, AtPhyB-PCM with PCB can be produced with a total assembly rate of 93 % and unsurprisingly, incorporation of BV with 37 % is much reduced. To tackle this deficit, we followed two strategies: (i) we increased expression time to 40 h to increase overall BV production; and (ii) most conventional phytochromes, including AtPhyB and DrBphP, form the Pr state as dark adapted state (Burgie und Vierstra 2014), therefore, we applied far-red light during expression to aid formation of the correct conformation of chromophore during assembly. In the end, both strategies boost assembly with BV, albeit application of far-red light has a more pronounced effect (Table 1). The combination of both approaches led to an astonishing total assembly rate with BV of about 84 %.

The absorbance spectra of AtPhyB-PCM with BV after red and far-red light illumination (Fig. 2a) reveal that the phytochrome typical Pr and Pfr states are formed at least spectroscopically correctly with absorption maxima at 663 nm and 724 nm, respectively. Surprisingly, the absorption maxima are only slightly red shifted, about 13 nm, compared to those of AtPhyB-PCM at 650 nm and 713 nm

(Fig. 2b) and much further blue shifted, about 20-40 nm, than the comparable bacterial phytochrome spectra with maxima around 700 nm and 750 nm (Fig. 2c).

Table 1: Expression conditions and assembly rates for purified AtPhyB-PCM with PCB or BV chromophore

chromophore	external addition ^a	expression time	far-red light ^b	assembly ratio (%) ^c
PCB	-	16 h	-	93
BV	-	40 h	+	84
BV	-	16 h	+	74
BV	+	16 h	-	62
BV	-	40 h	-	60
BV	-	16 h	-	37

^a The values refer to external chromophore addition during purification of AtPhyB-PCM

^b 800 nm (100 $\mu\text{W cm}^{-2}$) (Fig. S1).

^c As calculated from denatured protein spectra in 6.5 M guanidium hydrochloride.

The ratio of Pr/Pfr after red light illumination reveals that the red shift of AtPhyB-PCM with BV compared to PCB also causes more pronounced formation of the Pfr state (Table S1). When comparing thermal reversion of AtPhyB-PCM with PCB or BV with the reversion of the bacterial phytochrome *DrBphP*-PCM with BV we observed Pfr stability of BV-bound AtPhyB-PCM to be strongly decreased (Fig. 2d). The recovery rate was calculated using a three-component fit to eq (1) as described earlier (Bai et al. 2016; Takala et al. 2014b; Takala et al. 2015). Surprisingly, a model with only two exponentials was sufficient to describe the *DrBphP*-PCM thermal reversion data which is contrary to previous reports (Takala et al. 2014b; Takala et al. 2015). For BV-bound AtPhyB-PCM, the second longest time constant 213 min was the driving force for thermal reversion, while the main component for PCB-bound AtPhyB-PCM and *DrBphP*-PCM were the longest time constants 2773 min and 27657 min, respectively (Table S2). Intriguingly, both AtPhyB-PCM with PCB and BV share similar time constants for the shortest and longest component with about 12 min and 3000 min, respectively. Only the second time constant seems to be largely affected by the chromophore exchange and is reduced by half when comparing BV and PCB.

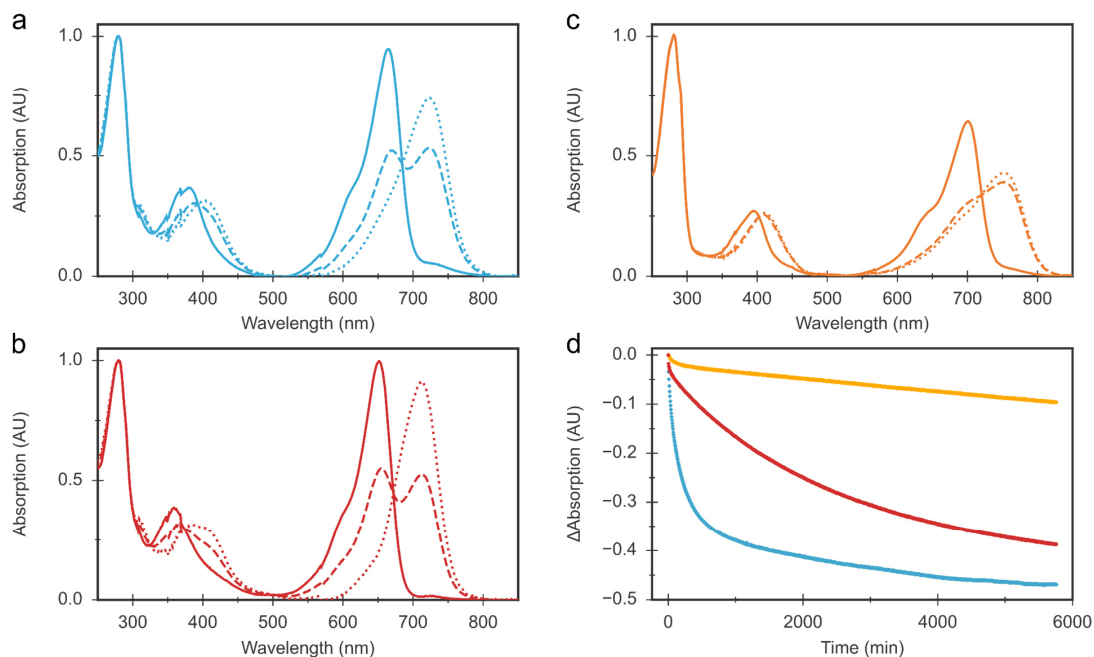


Figure 2: Spectroscopic analysis of different phytochromes and bilin chromophores. (a) AtPhyB-PCM with BV (blue) in the Pr state (solid line) after far-red illumination, mixed Pfr state (dashed line) after red light illumination and 100 % Pfr spectra (dotted line) calculated as described in (Butler et al. 1964). (b) as in (a) but AtPhyB-PCM with PCB (red). (c) as in (a) but DrBphP-PCM with BV (orange). (d) Thermal reversion of DrBphP-PCM with BV (orange), AtPhyB-PCM with BV (blue) or PCB (red).

An alternative binding mechanism for BV in plant phytochromes

The selected transient absorption data of AtPhyB-PCM with PCB or BV samples and DrBphP-PCM with BV after excitation at 620 nm, 620 nm and 660 nm, respectively, were measured. Transient absorption spectra typically consists of four overlapping contributions: (i) negative ground state bleaching due to the ground state population being removed by the pump pulse, (ii) negative stimulated emission arising from the interaction of the pump pulse with the excited state population thus being removed back to the ground state or its intermediates, (iii) the positive excited state absorption due to the transitions from the excited states, and (iv) the photoproduct absorption. The inverted steady-state absorption and fluorescence emission spectra of the studied samples are shown and scaled to the maximum of the transient species (Fig. 4a-c).

The bacteriophytochrome DrBphP-PCM shows typical spectral behavior after the excitation (Fig. 3a, 3d and S2), a stimulated emission prevails signals over 700 nm, while positive signal below 700 nm are attributed to the excited state absorption (Zienicke et al. 2013). After first couple of picoseconds, we observe the decay and a bathochromic shift of the negative signal. The amplitude of the negative signal steadily decreasing reflecting the decay of the fluorescent population of the excited states being returned to the ground state. At the late times of the experiment, in case of the DrBphP on the ns-timescale, the primary photoproduct spectrum, typically called Lumi-R, becomes visible.

Transient absorption spectra of AtPhyB-PCM with PCB show similar behavior (Fig. 3b, 3e and S2), apart from being blue shifted, if compared to the *DrBphP*-PCM spectra (Fig. 3a and 3d). The strongest negative signals around 650-700 nm are attributed to the mixture of stimulated emission and ground state bleaching. The negative signal around 680-740 nm has double band structure comparable to the bacteriophytochrome. Nevertheless, the 740 nm spectra band is assigned solely to the stimulated emission from the excited state as the ground state absorbance in this region is zero. The photoproduct absorption appears at a maximum of 685 nm at longer times.

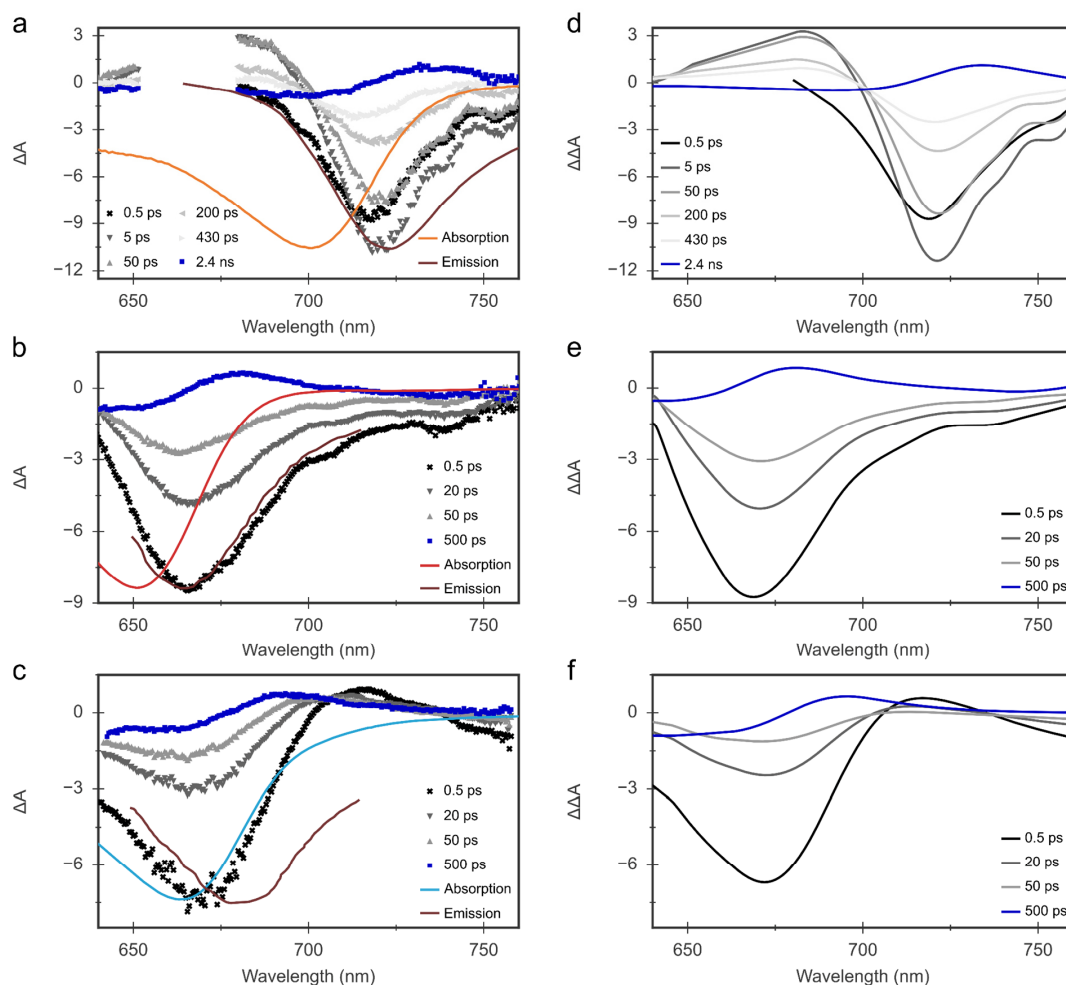


Figure 3: Transient absorption spectra of different phytochromes and bilin chromophores. Transient absorption spectra of (a, d) *DrBphP*-PCM with BV, (b, e) *AtPhyB*-PCM with PCB and (c, f) *AtPhyB*-PCM with BV were taken at different delay times after excitation. (a-c) Raw pump-probe data are presented as dots. In addition, inverted and scaled steady-state absorption and emission spectra are shown for comparison (solid lines). (d-f) Deconvoluted transient spectra are shown as solid lines. In each sample, the photoproduct spectrum (deep blue) is subtracted from the earlier spectra. The spectra highlight that photoproduct spectrum overlaps with a rather homogenous (non-productive) excited state spectra as early as the first picoseconds.

Differences are observed in transient absorption spectra of the phytochrome PCM sample binding either PCB (Fig. 3b and 3e) or BV (Fig. 3c and 3f). Shortly after excitation we can distinguish following contributions to the signal: (i) a strong negative signal around 650-680 nm overlapping

with the Pr spectra, thus it is attributed to the ground state bleaching; (ii) an excited state absorption can be observed as a positive signal only around 550 nm (Fig. S2); (iii) the photoproduct absorption spectra, positive signal at 690-700 nm, overlaps with the bleaching signal from Pr ground state and becomes dominant with the decrease of the bleaching signal over time. In conclusion, the difference between PCB and BV incorporated in AtPhyB-PCM lies in contribution of the stimulated emission reflecting the results obtained from the fluorescent experiments that this sample has a lower yield of fluorescent population (Fig. S2).

When comparing the strong spectral shift between AtPhyB-PCM with BV and DrBphP-PCM both in Pr and Pfr it appears unlikely that passive influence by surrounding amino acids is the driving force behind this shift as they tend to have only minor effects on the wavelength of the absorption maxima (Wagner et al. 2008). Furthermore, similarities between the Pr to Pfr conversion of AtPhyB-PCM with BV and PCB (Fig. 3) as well as their thermal reversion are rather strikingly (Table S2). Therefore, we conclude that a difference in the binding mechanism of the two phytochromes is the cause for the spectral properties. Reduced bilin chromophores, such as PCB and PΦB, are covalently attached via the cysteine 357 in AtPhyB which is a positionally conserved residue in plant phytochromes and cyanobacterial phytochromes (Burgie et al. 2014; Essen et al. 2008). However, the cysteine for covalent linkage in bacterial phytochromes is located in the PAS domain (Burgie et al. 2016; Takala et al. 2014a). As a result, two different types of binding mechanisms take place for the respective phytochrome families. The N-terminal located cysteine of bacterial phytochromes performs a nucleophilic attack on the C3² atom of the A ring which results in permutation of the delocalized π -electrons that causes an addition of a proton to the A ring (Fig. 4a). A similar mechanism occurs for plant and cyanobacterial phytochromes, however, bond formation takes place at the C3¹ atom of the A ring (Fig. 4b). Based on the binding mechanism for plant phytochromes, we propose that the nucleophilic attack of C357 in AtPhyB-PCM takes place at the C3¹ atom of BV. In fact, a similar target site is also proposed for an artificial create, bacterial-phytochrome based fluorescence protein with a blue shifted absorbance maxima (Hontani et al. 2016; Shcherbakova et al. 2015a). Contrary to the mechanism proposed for PCB or PΦB in plant phytochromes, permutation of the delocalized π electrons into the A ring cannot be performed for BV chromophore and a primary carbanion is generated which in turn must be protonated (Fig. 4c).

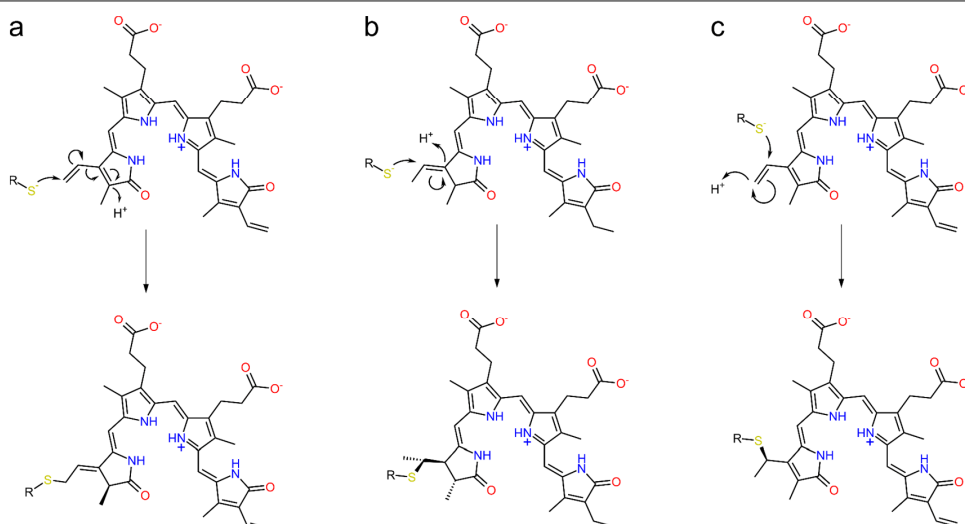


Figure 4: Covalent bond formation between different chromophores and phytochromes. (a) Nucleophilic attack of the cysteine in the PAS domain of bacterial phytochromes to the C3² atom of BV. (b) Bond formation between the positionally conserved cysteine in the GAF domain of plant phytochromes and the C3¹ atom of reduced bilin chromophores such as PCB. (c) Proposed mechanism for covalent bond formation between the conventional cysteine of plant phytochromes and BV.

Light-controlled protein-protein interaction is independent of AtPhyB chromophore

Since spectral analysis only reveals if the Pr and Pfr absorbance spectra are generated, a direct conclusion for structural rearrangement and thereby, biological function of the photoreceptor cannot be drawn that easily. To this end, we applied previously described protein-protein interaction assays for purified AtPhyB-PCM and PIF samples to test whether light-controlled interaction is still possible (Golonka et al. 2019). At first, we applied size exclusion chromatography (SEC) on mixtures of AtPhyB-PCM and PIF3(1-100) labeled with EYFP (P3.100-EYFP) after prior illumination with either red or far-red light. The usage of the EYFP moiety allowed parallel detection of P3.100 at 513nm and AtPhyB-PCM at 650 nm. Red light causes the formation of the Pfr state of AtPhyB-PCM which interacts tightly with P3.100-EYFP resulting in a shared elution volume for both proteins during SEC (Fig. 5a and 5d). The interaction was independent of the chromophore embedded in the AtPhyB-PCM. A fully functional AtPhyB-PCM needs to show light-dependent interaction with PIF, therefore, no interaction with the Pr state should occur. To this end, we illuminated the samples with far-red light before application to the SEC column. The elution profile shows clearly that both AtPhyB-PCM with BV and PCB in their Pr state elute independent of P3.100-EYFP (Fig. 5b and e). While SEC can only tell if interaction is occurring or not, we are also interested if the interaction strength of AtPhyB-PCM to P3.100-EYFP is altered as result of chromophore exchange. To quantify PhyB-PIF interaction, we applied fluorescence anisotropy measurement on the EYFP label of P3.100 in the presence of increasing concentration of AtPhyB-PCM either after red or far-red light illumination. Interaction between PIF and AtPhyB leads to an increase of the effective hydrodynamic radius. Larger particles tend to have a slower rotational diffusion in solution which in turn results in a higher fluorescence anisotropy value. Similar to the SEC results, P3.100-EYFP

displays tight interaction with the Pfr state of both AtPhyB-PCM with BV or PCB (Fig. 5c and f). The ratios of Pr to Pfr have to be considered when determining dissociation constant K_D of the Pfr interaction as red illumination causes the formation of a Pr/Pfr state mixture (Table S1). Taken this into account, we could determine an interaction strength between P3.100 and AtPhyB-PCM with BV or PCB of about (250 ± 40) nM and (200 ± 70) nM, respectively. In direct comparison, Pr state interaction with P3.100-EYFP was strongly reduced and could only be roughly extrapolated from the data. Both AtPhyB-PCM with BV and PCB in the Pr state displayed weak interaction with P3.100-EYFP in the low micromolar range of $> 5 \mu\text{M}$ and $> 2 \mu\text{M}$, respectively.

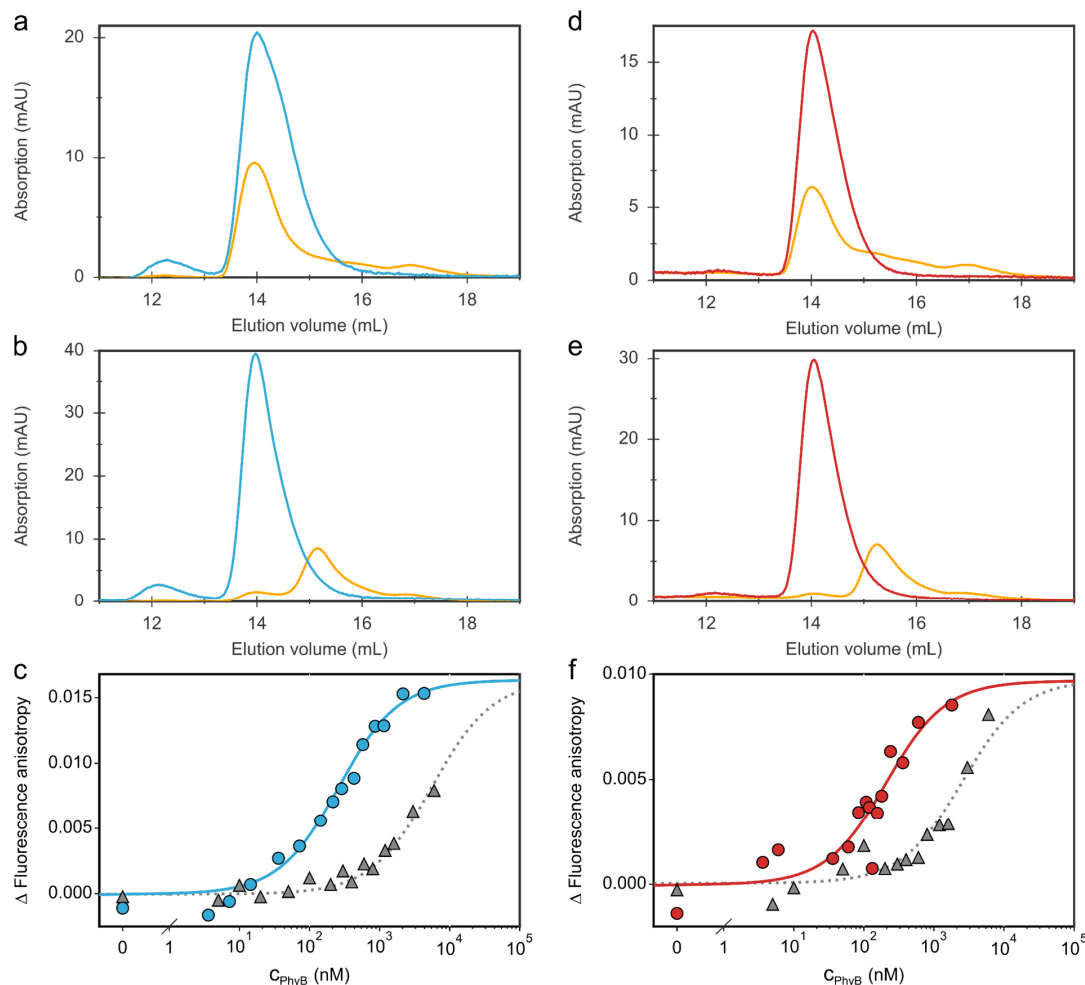


Figure 5: Interaction studies on AtPhyB-PCM and P3.100-EYFP. (a) Size exclusion analysis on AtPhyB-PCM with BV (blue) and P3.100-EYFP (yellow) mixtures after red light illumination. (b) as in (c) but with prior far-red illumination. (c) Fluorescence anisotropy of P3.100-EYFP and increasing concentrations of AtPhyB-PCM with BV either in the Pr (blue) or Pfr state (gray). (d) Size exclusion measurements on AtPhyB-PCM with PCB (red) and P3.100-EYFP (yellow) mixtures after red light illumination. (e) as in (d) but with prior far-red illumination. (f) Fluorescence anisotropy of P3.100-EYFP and increasing concentrations of AtPhyB-PCM with BV either in the Pr (red) or Pfr state (gray).

In conclusion, the assays reveal strikingly that AtPhyB-PCM with BV interacts with its partner protein P3.100 in a light-dependent manner that is identical to AtPhyB-PCM with PCB (Fig. 5).

Furthermore, interaction strength of the Pfr state for PCB-bound or BV-bound AtPhyB-PCM with P3.100 is nearly identical. The Pr state interaction is strongly reduced compared to the Pfr state interaction.

Alteration of BV assembly rate in plant phytochrome

Based on our proposed mechanism for BV binding in AtPhyB-PCM, we introduced the mutations H355S, R578K and R578H into AtPhyB-PCM with the goal to disrupt or increase the BV incorporation. In theory, H355 might form a catalytic dyad with C357 that might support bond formation to bilins (Fig. 6a). Similar observations were made for PhyA previously when another histidine (H358 in AtPhyB) directly neighboring the cystine for covalent attachment was substituted with other amino acids leading to a drop in chromophore incorporation (Bhoo et al. 1997). In fact, the H355S mutant of AtPhyB-PCM showed a decrease in assembly rate with BV of 66 % compared to wildtype AtPhyB-PCM purified under the same conditions achieving 75 %, which hints towards at least a minor role of H355 during covalent bond formation. The amino acid arginine 578 located in the tongue region of AtPhyB-PCM is in close proximity to the C3² atom of the bilin chromophore (Fig. 6a). Arginine already provides a positive charge; however, this charge is delocalized between two amines. Therefore, replacing R578 by lysine might lead to more focused charge distribution which might aid electron-withdrawing towards the C3² atom of the bilin. Alternatively, the substitution of R578 by histidine provides a proton donor near the C3² atom that might support the incorporation of BV while retaining the positive charge. Introduction of R578K into AtPhyB-PCM yielded an assembly rate of 74 % and therefore, had no effect on BV incorporation. AtPhyB-PCM with R578H, denoted biliverdin-binding phytochrome B (BVB-PhyB), increased assembly rate with BV to 87 %, resulting in the highest assembly rate with BV for AtPhyB-PCM to date.

All AtPhyB-PCM variants retain their ability to form the Pr and the Pfr state spectroscopically (Fig. 6b-d) with ratios similar to AtPhyB-PCM with BV (Table S1). However, absorption maxima for the H355S variant are slightly blue shifted both, about 5 nm, for the Pr at 659 nm and the Pfr at 718 nm compared to wildtype and R578 mutants. Surprisingly, absorption in the red to far-red spectra for the R578K variant and BVB-PhyB were strongly increased compared to the wildtype. However, similar observations were made during mutational studies on the chromophore binding pocket of phytochromes indicating to alterations of bilin conformation independent of the Z to E isomerization of C15=C16 (Wagner et al. 2008). Thermal reversion of BVB-PhyB (Fig. 6e) was dominated by the second longest time constant of 132 min (Table S2) and intriguingly, the longest time constant was reduced by two thirds compared to the wildtype AtPhyB-PCM with BV. Comparison of the other AtPhyB-PCM mutants and the wildtype AtPhyB-PCM with BV revealed that all time constants were increased in these cases (Table S2). The thermal reversion for the R578K variant was controlled by the fastest time constant of 85 min which is seven times slower than observed for the wildtype. The H355S, however, displayed a thermal reversion dominated by the longest time constant (Table S2). In conclusion, alterations around the A-ring of the chromophore can influence thermal reversion tremendously.

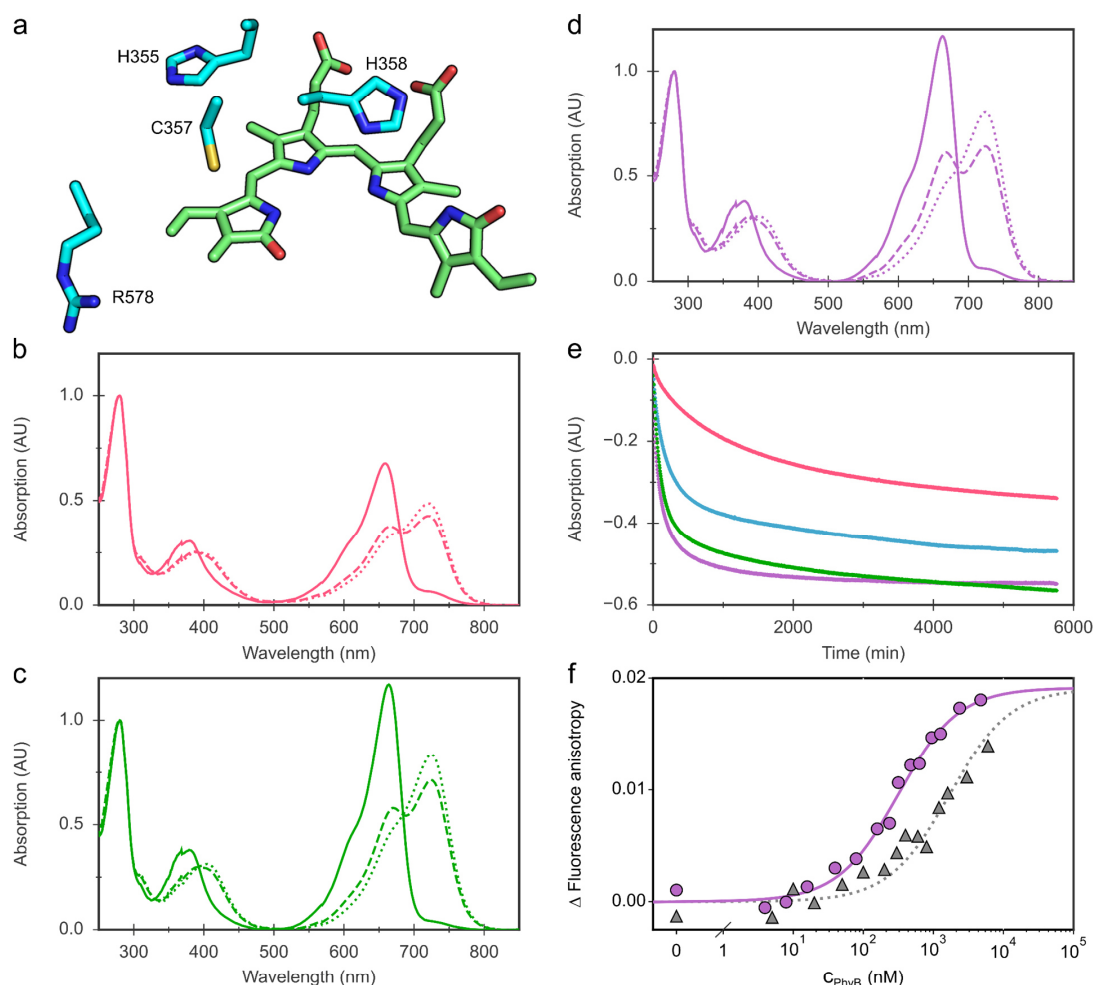


Figure 6: Alteration of AtPhyB-PCM with BV by mutational studies. (a) Residues in close proximity to the cysteine 357 and the A-ring of the bilin (pdb: 4our). (b) AtPhyB-PCM with H355S (magenta) in the Pr state (solid line) after far-red illumination, mixed Pfr state (dashed line) after red light illumination and 100 % Pfr spectra (dotted line) calculated as described in (Butler et al. 1964). (c) as in (b) but AtPhyB-PCM with R578K (green). (d) as in (b) but AtPhyB-PCM with R578H, denoted BVB-PhyB, (purple). (e) Thermal reversion of AtPhyB-PCM with BV (blue) and BVB-PhyB (purple). (f) Fluorescence anisotropy of P3.100-EYFP and increasing concentrations of AtPhyB-PCM with BV either in the Pr (blue) or Pfr state (gray).

When tested for light dependent interaction with P3.100, BVB-PhyB revealed to possess an interaction strength of (300 ± 40) nM and $>1 \mu\text{M}$ for the Pr and Pfr state, respectively (Fig. 6f), which is in close approximation to the K_D values measured for AtPhyB-PCM with either PCB or BV (see above).

Discussion

In this study, we could show that AtPhyB-PCM, contrary to previous reports (Li und Lagarias 1992; Rockwell und Lagarias 2010), can functionally incorporate BV while retaining light-dependent interaction with P3.100. While the modification of cyanobacterial phytochromes was rather extensive to achieve BV incorporation (Fushimi et al. 2019), AtPhyB-PCM displayed a high assembly rate with BV even without the introduction of mutations (Table 1). The assembly with BV in *E.coli*

cells modified to overexpress HO1 as well as *AtPhyB-PCM* can be boosted by increasing the expression time and/or applying far-red light during expression. The positive effect of prolonged expression might be attributed to reduced BV release from HO1 in the absence of biliverdin reductase (Liu und Ortiz de Montellano 2000). Hence, HO1 releases BV to the surrounding cytosol with decreased speed which might be an additional cause of poor BV uptake by *AtPhyB-PCM*. While we contribute the positive effect of far-red light on incorporation of BV into *AtPhyB-PCM* to a support role during formation of the dark adapted Pr state (Burgie und Vierstra 2014). We note that far-red light might increase temperature during expression, however, this hypothesis appears rather farfetched as cells are kept under constant temperature control.

The main difference between *AtPhyB-PCM* with BV and PCB seems to be encoded in the spectral properties rather than its function during protein-protein interaction. On first glance, BV-bound and PCB-bound *AtPhyB-PCM* share large similarities in absorption spectra for Pr and Pfr state, time constants during thermal reversion and transient absorption spectra when compared to *DrBphP-PCM*. However, differences between the two chromophores are visible such as spectral shifts of the absorption maxima and different amplitudes during thermal reversion. Especially when comparing transient absorption spectra, it becomes obvious that the contribution of stimulated emission is much higher for PCB as it is for BV.

Essentially in all ultrafast spectroscopic studies of phytochromes (Choudry et al. 2018; Ihalainen et al. 2018; Kennis und Groot 2007) the transient absorption data is analyzed globally either as a parallel decay model or as a sequential model, where the excited state decays towards the photoproduct. In typical models, the excited state decays either monotonously having a single time constant or multiple time constants. Thus, the rate of the photo product, Lumi-R state, becomes to be determined by the decay of the excited state product(s) of the chromophore molecule, typically in the order of tens to hundreds of ps. This contrasts to the recent observation by SFX where an ultrafast isomerization product is observed at 1 ps timescale (Claesson et al. 2020). However, if the photoproduct, which has rather small contribution to the overall excited state spectrum, would be produced in the very early timescale after the excitation, it will be eliminated by the non-productive excited state spectrum. We note that the Lumi-R spectra term used in this section implies the spectra that is evolved from the “pure” Lumi-R and a ground state bleach that indicates the population that has been removed permanently from the ground state to form photoproduct. In this way, we distinguish a so-called productive pathway from the non-productive pathway (Fig. 3d-f). In each case, a steady decay of the non-productive path can be discerned, and their spectral contributions and lifetimes can be determined. Here, we are unable to untangle the very early times spectra without a contribution of the productive spectrum, due to coherent artefacts that arise from the spatial and temporal overlap of the two laser pulses in highly absorptive sample at the time of sample excitation (Dietzek et al. 2007). In summary, our analysis suggests that the parallel evolution of the photoproduct and unproductive excited state dynamics (both, fluorescent and non-fluorescent populations) happen at very first picoseconds.

The herein, proposed mechanism for BV attachment in plant phytochromes has at least three disadvantages compared to the general mechanisms with reduced bilins: (i) the formation of a positive charge at the C3¹ atom is not supported by the BV chromophore as strong electron-withdrawing groups next to the C3¹ atom are missing and electrons cannot permute into the A ring, (ii) the carbanion at the C3² position can only be generated if a positively charged amino acid is in close proximity, and (iii) protonation at the C3² atom has to take place which hints towards a pH dependency of the reaction. Taken together, these drawbacks may explain why BV is rather poorly incorporated into plant phytochromes compared to reduced bilins, such as PCB and PΦB, and was not reported earlier. When searching for a way to improve BV incorporation into AtPhyB it appears that only two options might be suitable: (i) alteration of the pH value of the surrounding matrix or (ii) introduction of mutations into AtPhyB-PCM to aid BV binding. While the first approach appears suitable for *in vitro* application, it is close to be impossible to alter intracellular pH without causing significant changes the metabolism and behavior of cells (Putnam 2012). As a result, only the second approach is suitable for future application in mammalian cell culture or model organisms, however, room for improvement appears rather limited. For one thing, only the tongue region is in close proximity to the C3² atom of the bilin chromophore and for another, this tongue region presumably undergoes large structural changes between Pr and Pfr conformation (Takala et al. 2014a). At the moment, only the Pr conformation of AtPhyB-PCM is known (Burgie et al. 2014) and therefore, structure guided modification can only be performed based on the Pr state structure. With this in mind and with the knowledge of Pr being the dark-adapted state (Burgie und Vierstra 2014), we could only identify the position R578 to be suitable, hence yielding BVB-PhyB which we hope to contribute largely in application of the AtPhyB-PIF system in optogenetics and biotechnology without external chromophore addition.

Material & Methods

Molecular biology and protein expression

The plasmid pDG282 (Golonka et al. 2019) which encodes *A. thaliana* PhyB (1-651), *Synechocystis* sp. heme oxygenase 1 and pcyA was used for production of AtPhyB-PCM with phycocyanobilin chromophore (Mukougawa et al. 2006). For assembly of AtPhyB-PCM with biliverdin, pDG282 was modified by removal of the gene for pcyA yielding the plasmid pDG459. The AtPhyB PCM mutants H355S and R578H were generated by introducing mutations into the plasmid pDG459 via side-directed mutagenesis resulting in the plasmids pDG468 and pLH052. The photosensory core module of bacterial phytochrome of *D. radiodurans* BphP (1-512) was subcloned into pDG459 such that the gene for AtPhyB-PCM was exchanged for DrBphP-PCM. The plasmid pDG171 which contains the AtPIF3 (1-100) N-terminally tagged with His₆-SUMO (Panavas et al. 2009) and C-terminally fused to EYFP, attached via a DSAGSAGSAG linker, was utilized for P3.100-EYFP preparation as described previously (Golonka et al. 2019).

Protein expression of AtPhyB-PCM and DrBphP-PCM was carried out in *Escherichia coli* LOBSTR cells (Andersen et al. 2013), which were grown in terrific broth (TB) medium, supplemented with

100 $\mu\text{g mL}^{-1}$ streptomycin at 37 °C in darkness. At an optical density at 600 nm (OD_{600}) of 0.7-0.9, cells were complemented with δ -aminolevulinic acid to a concentration of 0.5 mM and induction was performed by addition of 1 mM β -D-a-thiogalactopyranoside (IPTG). The cultivation was carried on at 18 °C. For AtPhyB-PCM with PCB and for DrBphP-PCM, expression was conducted for about 16 h in darkness, whereas for AtPhyB-PCM with BV the time given for expression was either 16 h or 40 h and cells were kept in darkness or under 800 nm ($100 \mu\text{W cm}^{-2}$) far-red light conditions. Expression for comparison of AtPhyB-PCM wildtype and mutants was performed for 40 h without far-red supply and no external chromophore was added during purification.

Cell harvest was performed by centrifugation and the cell pellet was resuspended in lysis buffer [50 mM Tris/HCl pH 8.0, 20 mM NaCl, 20 mM imidazole; supplemented with protease inhibitor mix (cOmplete Ultra, Roche Diagnostic, Mannheim, Germany) and 20 $\mu\text{g mL}^{-1}$ DNase I (PanReac AppliChem, Darmstadt, Germany)]. Cells were lysed by sonification and cleared by centrifugation. Only in the first reconstitution experiment with AtPhyB PCM, cleared lysate was supplemented with 100 mM biliverdin hydrochloride (Frontier Scientific, Logan, Utah, United States) and incubated for 1 h at 4 °C. Purification was performed via immobilized ion affinity chromatography (IMAC) on Protino Ni-NTA columns (Macherey-Nagel, Düren, Germany) utilizing a 20 to 500 mM imidazole gradient. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) supplemented with 1 mM Zn^{2+} was used to analyze elution fractions for covalently bound bilin chromophores by zinc-induced fluorescence (Berkelman und Lagarias 1986). Fractions containing phytochrome with bilin were pooled, incubated with protease inhibitor mix (cOmplete Ultra, Roche Diagnostic, Mannheim, Germany) for 1h and dialyzed overnight into AEX buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl, 5mM 2-mercaptoethanol). The sample was applied to a HiTrap Q HP 1 mL anion-exchange column (GE Healthcare Europe GmbH, Freiburg, Germany), and eluted via a two-step linear gradient from 50 to 300 mM NaCl and 300 mM to 500 mM. Eluted fractions were analyzed by SDS-PAGE, before pooling pure fractions and dialysis against storage buffer [10 mM Tris/HCl pH 8.0, 300 mM NaCl, 10 % glycerol]. Protein samples were stored at -80 °C.

Protein expression and purification of AtP3.100 was performed as described before (Golonka et al. 2019).

Spectroscopic analysis

The purified proteins were analyzed at 22°C by absorption spectroscopy on an Agilent 8453 and a Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany). Concentrations of P3.100-EYFP and AtPhyB-PCM with PCB were determined using the extinction coefficients of $84300 \text{ M}^{-1} \text{ cm}^{-1}$ at 513 nm (Shaner et al. 2005) and of $47600 \text{ M}^{-1} \text{ cm}^{-1}$ at 672 nm (Golonka et al. 2019), respectively. Absorption spectra of AtPhyB-PCM with BV and DrBphP-PCM were also recorded in 6.5 M guanidinium hydrochloride and concentration of protein and bilin was calculated from absorption of tryptophan at 280 nm and absorption of BV at 380 nm with the respective extinction coefficient of $39000 \text{ M}^{-1} \text{ cm}^{-1}$ for BV (Gasser 2015). Pr $\leftrightarrow$ Pfr conversion of AtPhyB PCM with PCB or with BV was recorded after prior illumination with light-emitting diodes

(LEDs) with emission maxima of (640 ± 15) nm and (750 ± 15) nm, respectively. The Pr/Pfr ratios upon 640 nm and 750 nm illumination and the spectrum of the pure Pfr state were calculated as described in (Butler et al. 1964). (670 ± 15) nm and (780 ± 15) nm light was utilized for Pr ↔ Pfr conversion of *DrBphP*-PCM and determination of Pr/Pfr ratios. The thermal recovery of the phytochromes was recorded on a Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies) after 640 nm illumination at 22 °C for 4 days and data was fitted to equation (1) using the Fit-o-mat software (Möglich 2018):

$$A_{\text{Pfr/Pr}} = A_0 + A_1 \times e^{\left(-\frac{t}{\tau_1}\right)} + A_2 \times e^{\left(-\frac{t}{\tau_2}\right)} + A_3 \times e^{\left(-\frac{t}{\tau_3}\right)} \quad (1)$$

where $A_{\text{Pfr/Pr}}$ represents the ratio of the absorption at the maxima of the Pfr to the Pr state, A_n is the decay amplitude, τ_n represents the time constant of the respective decay and t is the time.

Ultrafast pump-probe spectroscopy

Transient absorption experiments were performed on a home build setup based on a Ti:sapphire femtosecond laser system (1kHz, 800 nm). The main beam was split into pump and probe beams. The pump beam was sent through the two-stage home build noncollinear optical parametric amplifier to produce tuned beam at specified central wavelength on the first stage (λ_{exc} =660nm and 620; 10 nm bandwidth) and amplified by the second stage. The probe beam was focused on a 2 mm sapphire plate to generate broadband (420-800 nm) white light continuum which was split by 50/50 beam splitter to reference and probe beams. The probe beam was focused on a flow cell cuvette where it was overlapped with the pump beam. The sample was flowing continuously into an enclosed reservoir where it was illuminated with a LED (772 nm and 720 nm for bacteriophytochrome and plant phytochrome samples, respectively) to ensure that all of the population is back to the Pr state before it is being delivered to the beam area. The mutual polarization of the pump and probe beams was set to the magic angle (54.7) by Berek compensator to avoid anisotropy effects. All measurements were carried out at room temperature. Time-resolved absorption changes were measured by detecting probe and reference beams dispersed on the double-diode array. The difference spectra between excited and unexcited sample was generated by chopped pump beam at 500 Hz. The time delay between pump and probe pulses was set by a computer-controlled delay line placed in the probe beam path. Absorption changes were observed in time window from 0 to 3.5 ns. The time resolution of the setup was limited by the laser pulse width, and in current configuration was estimated to be about 200 fs.

Size-exclusion chromatography

A Superdex 200 Increase 10/300 GL (GE Healthcare) column on a ÄKTApure system, equipped with a multi-wavelength detector (GE Healthcare) was used to analyze light-dependent *AtPhyB*-PCM and P3.100-EYFP interaction. A mixture of 50 µM *AtPhyB*-PCM and 10 µM P3.100-EYFP in phosphate buffer (67 mM sodium phosphate buffer pH 8.0 and 200 mM NaCl) was illuminated with 650 nm or 720 nm light for 2 min and thereafter, 25 µL were applied onto the column at a constant flow rate

of 0.75 mL min⁻¹. Absorption was recorded at 513 nm and 650 nm for P3.100-EYFP and AtPhyB-PCM, respectively.

Fluorescence anisotropy measurements

20 nM P3.100-EYFP and up to 6 µM of AtPhyB-PCM, which was illuminated either with 640 nm or 750 nm beforehand, were prepared in spectral buffer (20 mM HEPES/HCl pH 7.3, 10 mM NaCl and 100 µg mL⁻¹ bovine serum albumin). Samples were transferred into black 384-well MTPs (Brand, Wertheim, Germany) and illuminated with 640 nm or 750 nm before measurement on a CLARIOstar multimode microplate reader (BMG Labtech, Ortenberg, Germany). Fluorescence was recorded at excitation and emission wavelengths of (482 ± 16) nm and (530 ± 40) nm utilizing a 504-nm long-pass dichroic mirror. Gain adjustment for the vertical and horizontal detection was performed to yield fluorescence anisotropy signal for P3.100-EYFP of 0.315 determined previously (Golonka et al. 2019). Anisotropy data was fitted to equation (2) using the Fit-o-mat software (Möglich 2018):

$$r = r_0 + \frac{r_1}{2} \times \left\{ 1 + \frac{[AtPhyB]}{c_{tot}} + \frac{K_D}{c_{tot}} - \sqrt{\left(1 + \frac{[AtPhyB]}{c_{tot}} + \frac{K_D}{c_{tot}} \right)^2 / 4 - \frac{[AtPhyB]}{c_{tot}}} \right\} \quad (2)$$

where r represents the anisotropy of P3.100-EYFP, $[AtPhyB]$ is the concentration of AtPhyB-PCM in the Pr or Pfr state, $c_{tot} = 20$ nM is the concentration of P3.100-EYFP, and K_D is the dissociation constant.

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Supplemental

Table S1: Pr to Pfr ratios after 640 nm (red) or 750 nm (far-red) illumination for AtPhyB-PCM (WT) with PCB or BV, AtPhyB-PCM mutants and DrBphP-PCM with BV determined as described in (Butler et al. 1964).

protein	bilin	red		far-red	
		Pr (%)	Pfr (%)	Pr (%)	Pfr (%)
AtPhyB-WT	PCB	40	60	95	5
AtPhyB-WT	BV	28	72	93	7
DrBphP-WT	BV	10	90	94	6
AtPhyB-H355S	BV	14	86	98	2
AtPhyB-R578K	BV	15	85	98	2
AtPhyB-R578H	BV	21	79	97	3

Table S2: Time constants τ and decay amplitudes A for thermal reversion of AtPhyB-PCM and DrBphP-PCM with different chromophores after 640 nm light absorption.

protein	bilin	τ_1 (min)	A_1 (%)	τ_2 (min)	A_2 (%)	τ_3 (min)	A_3 (%)
AtPhyB-WT	PCB	12.35	6.8	526.3	5.4	2773	87
AtPhyB-WT	BV	12.94	16	212.8	50	3041	32
DrBphP-WT	BV	-	-	96.15	4.5	27657	95
AtPhyB-H355S	BV	57.14	8.9	714.3	36	3518	53
AtPhyB-R578K	BV	85.47	57	454.5	17	4054	24
AtPhyB-R578H	BV	12.71	28	131.6	51	1002	19

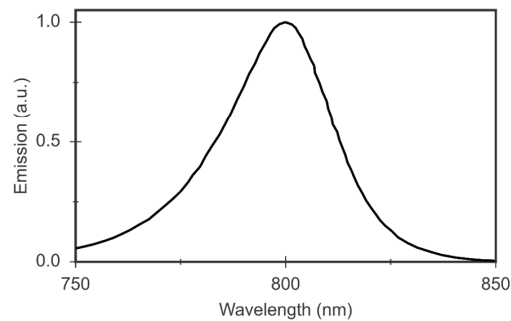


Figure S1: Emission spectra of 800 nm LEDs utilized during protein expression.

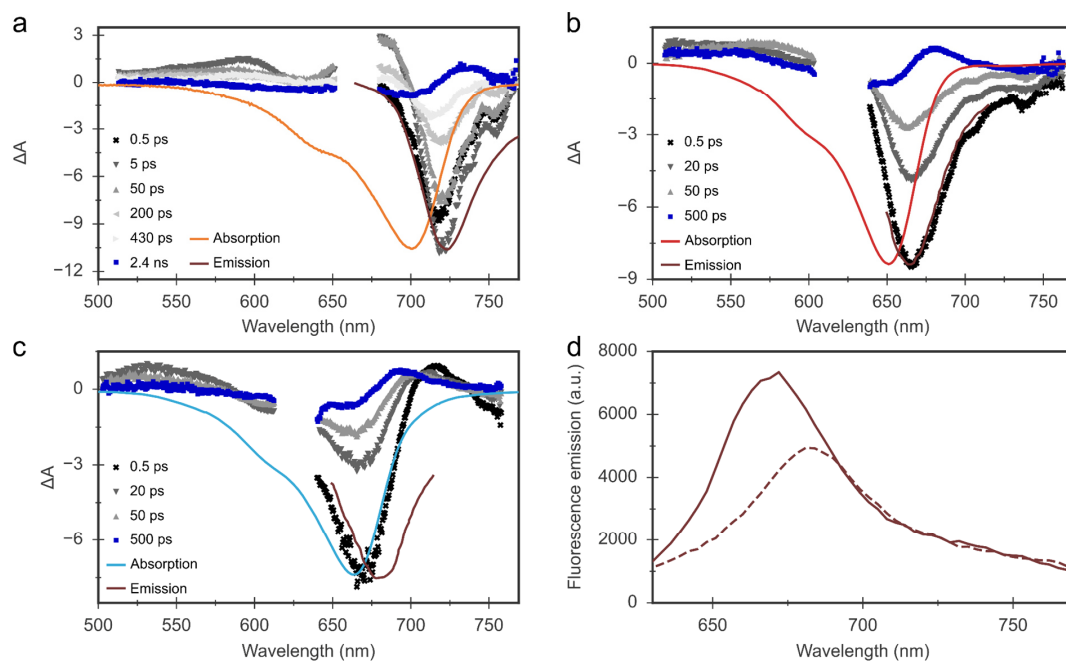


Figure S1: Transient absorption spectra of different phytochromes and bilin chromophores. (a) *DrBphP*-PCM with BV, (b) *AtPhyB*-PCM with BV, and (c) *AtPhyB*-PCM with PCB transient absorption spectra were taken at different delay times after excitation. Raw pump-probe data are presented as dots. In addition, inverted and scaled steady-state absorption and emission spectra are shown for comparison (thin solid lines). (d) Fluorescence emission spectra of *AtPhyB*-PCM with PCB (solid line) and BV (dashed line).

5.4 Manuskript IV

Titel: „Alternative red-light inducible split transcription factor system”

Autoren: Fischbach, P.; Golonka, D.; Fedotov, S.; Hiltbrunner, A.; Möglich. A., Zurbriggen
M. D.

Noch nicht eingereicht

Alternative red-light inducible split-transcription-factor systems

Patrick Fischbach¹, David Golonka², Sergey Fedotov³, Andreas Hiltbrunner⁴, Andreas Möglich^{2*}, Matias D. Zurbriggen^{1*}

¹Institute of Synthetic Biology, University of Düsseldorf and CEPLAS, 40225 Düsseldorf, Germany

²Lehrstuhl für Biochemie, Photobiochemie, Universität Bayreuth, 95447 Bayreuth, Germany

⁴ Institute of Biology II, Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany.

* Corresponding author: Email: Andreas.Moeglich@uni-bayreuth.de ; matias.zurbriggen@uni-duesseldorf.de;

Abstract

The red-light induced phytochrome B underpins a split-transcription-factor system serving as an efficient optogenetic tool for the control of gene expression. The system is based on the light-dependent interaction of *Arabidopsis thaliana* phytochrome B and its interacting factors. To enable the control of multiple intracellular processes in parallel by different phytochromes, we engineered an alternative red-light inducible split transcription factor system based on *A. thaliana* phytochrome A and a novel light dependent interactor OPA (only phytochrome A). The new system shows similar behavior, reversibility and sensitivity as the known phytochrome B-based while being completely orthogonal. The novel phytochrome A based system expands the optogenetic toolbox and thus paves the way towards multi-pathway control with a single wavelength.

Keywords

gene expression; optogenetics; phytochrome; protein:protein interaction; sensory photoreceptor; signal transduction

Introduction

Sensory photoreceptors are light-sensing proteins found in a wide range of organisms such as bacteria, fungi, animals and plants. The biological role of these sensors is to detect environmental light conditions and to alter behavior and physiology of organisms accordingly (*Jékely, 2009; Whippo and Hangarter, 2006*). Owing to the reversibility and spatiotemporal precision of the processes elicited by these proteins, photoreceptors have been widely applied in synthetic biology to control cellular processes and state by light (*Chernov et al., 2017; Losi et al., 2018*), an approach called optogenetics (*Deisseroth et al., 2006*). As red and far-red light exhibits deeper penetration of biological tissue than light of shorter wavelengths (*Jöbsis, 1977; Weissleder and Ntziachristos, 2003*), photoreceptors sensitive to these regions of the electromagnetic spectrum are of great interest for optogenetics. The red-light-sensitive photoreceptor that arguably has been most extensively deployed in optogenetics is phytochrome B of *Arabidopsis thaliana* (PhyB). In nature, PhyB controls a range of important responses to red and far-red light, including shade avoidance, onset of germination and photomorphogenesis (*Kreslavski et al., 2018; Pham et al., 2018*). At the molecular level, these responses are mediated via light-controlled protein-protein interactions (PPI) with partner proteins such as the phytochrome interacting factors (PIFs) (*Pham et al., 2018*).

Phytochromes generally use linear tetrapyrrole chromophores, bilins, to sense red and far-red light (*Nagano, 2016*). The domain architecture and type of bilin of the phytochrome varies depending on the organism of origin (*Rockwell and Lagarias, 2010*). Plant phytochromes consist of an N-terminal photosensor module (PSM) and a C-terminal output module (OPM). The PSM comprises PAS (Per-Arnt-Sim) (*Möglich et al., 2009*), GAF (cGMP-stimulated phosphodiesterase, *Anabaena* adenylate cyclases, FhlA) (*Aravind and Ponting, 1997*) and PHY (phytochrome-specific) domains (*Essen et al., 2008*). An N-terminal extension (NTE) precedes the PSM and is required for light-dependent interactions with PIF proteins. The OPM is involved in mediating many of the physiological responses of phytochromes and comprises two PAS domains and a histidine kinase-related domain (HKRD) with reported serine/threonine-kinase activity (*Yeh and Lagarias, 1998*). The GAF domain holds the plant-specific chromophore phytochromobilin (PΦB), that is covalently bound as a thioether to a cysteine residue within the GAF domain (*Rockwell and Lagarias, 2010*). Although plant phytochromes naturally employ PΦB, they can be functionally reconstituted with phycocyanobilin (PCB) (*Kyriakakis et al., 2018; Mroginski et al., 2011*), a cyanobacterial chromophore. As PCB can be extracted from cyanobacteria and is commercially available, applications of plant phytochromes in mammalian cells commonly rely on exogenous PCB supplementation. Phytochromes can populate the red light absorbing Pr and far-red light absorbing Pfr states which differ in the configuration of their bilin chromophores around the C15–C16 double bond (*Li et al., 2011*). Whereas in canonical phytochromes the Pr state is most stable and assumed in darkness, for bathyphytochromes it is the

Pfr state. Red light drives the isomerization from the 15Z configuration in Pr to 15E in Pfr, which results in a bathochromic shift of the absorption spectrum (*Li et al., 2011*). Thermal reversion in the dark or application of far-red light return the chromophore to the Pr conformation (*Rockwell and Lagarias, 2010*). As first resolved for bacterial phytochromes, the conversion between Pr and Pfr is linked to conformational transitions within the protein that propagate downstream to the effector (*Takala et al., 2014*). Although the precise molecular mechanism awaits further study, in plant phytochromes these changes underpin the light-dependent PPIs with PIFs and other partner proteins (*Ni et al., 1998*). Specifically, the PIF proteins interact preferentially with the Pfr state of phytochromes (*Golonka et al., 2019; Khanna et al., 2004; Leivar and Quail, 2011*). This interaction is mediated by an ‘active phytochrome B binding’ (APB) motif embedded within the N-terminal 100 amino acids of the PIF proteins (*Golonka et al., 2019; Khanna et al., 2004*). Likewise, only the N-terminal part of PhyB, encompassing its NTE and the PSM, is sufficient for light-activated, photoreversible interaction with the PIFs (*Levskaya et al., 2009; Matsushita et al., 2003*). Therefore, applications in optogenetics rely on truncated N-terminal fragments of both the phytochrome and the PIFs (*Beyer et al., 2015; Toettcher et al., 2011b*). These applications generally harness the photoreversible PPI between phytochromes and PIF to bring into spatial proximity target proteins and thereby activate them on light cues. As opposed to other light-activated PPIs, the PhyB:PIF interaction can be bimodally toggled on and off by red and far-red light, thus achieving superior temporal resolution and dosability. Moreover, certain PIF variants exhibit a remarkably stringent light response with an at least thousand-fold affinity difference for PhyB under red and far-red light, respectively (*Golonka et al., 2019*). Owing to these benefits, multiple optogenetic switches based on the PhyB:PIF PPI have been engineered and applied to control cellular processes, such as gene expression, protein localization and signal pathways (*Müller et al., 2013a; Shimizu-Sato et al., 2002; Toettcher et al., 2011; Toettcher et al., 2013*). A prominent example is the red-light inducible split-transcription-factor system (*Müller et al., 2013a*). In this system the PhyB-PSM is fused to a VP-16 transactivation domain, and the ABP-motif of PIF3 or PIF6 is connected to a tetracycline repressor (tetR). The tetR binds to the tetracycline resistance operon tetO, situated in close proximity to a minimal promoter which governs expression of a reporter. Red light illumination triggers association of PhyB and PIF, thus resulting in the localization of VP-16 and the minimal promoter, which in turn activates gene expression. Notably, the system can be repeatedly activated and inactivated by applying red and far-red light, respectively (*Müller et al., 2013a*).

Transcending the existing split transcription factor system, we aimed to devise a version that utilizes *A. thaliana* phytochrome A (PhyA) instead of PhyB, while retaining exquisite light responsiveness. To prevent crosstalk with the PhyB:PIF PPI, a partner protein is needed that solely interacts with PhyA. However, the PhyA interactors known and characterized to date are not specific but also

interact with other phytochromes, especially PhyB. As a case in point, PIF1 and PIF3 interact with PhyA in a light-dependent manner via an active phytochrome A binding (APA) motif (*Leivar and Quail, 2011*) but also harbor an APB motif that allows interactions with PhyB (*Pham et al., 2018*). Likewise, other PhyA interaction partners currently identified, such as SPA1, also display cross-reactivity with PhyB (*Lu et al., 2015; Sheerin et al., 2015*). To surmount this fundamental limitation, we employed the split transcription factor system to assess for light dependent interaction between PhyA and several candidate interaction partners, originating from a genetic screen. We thus identified one protein of unknown function (AT1G48770), denoted OPA, which is interacting with PhyA in a light dependent manner but not with PhyB. The split transcription factor system based on PhyA and OPA exhibited response kinetics comparable to those of the original one. However, the light sensitivity was slightly lower which we attribute to a different binding affinity of the PhyA:OPA compared to the PhyB:PIF system. The new PhyA-based system works orthogonally to the PhyB-based one, thus auguring the combination of the two systems for the regulation of separate cellular processes with one wavelength.

Results

A yeast two-hybrid screen for novel light-dependent PPIs in *A. thaliana* identified a cohort of candidate proteins apparently interacting with PhyA in light-dependent manner (*Fedotov & Hiltbrunner in preparation*). To analyze the interaction between PhyA and the candidate interactors in dependence of light, we designed a split transcription factor system akin to a previously reported one based on PhyB:PIF (*Müller et al., 2013a*). To this end, PhyA was fused to a viral trans activator VP-16 domain, and the candidate interacting partners were fused to the tetR transcription factor (Fig.1). Provided interaction between the two entities takes place, the trans activator colocalizes with the synthetic minimal promoter and thereby induces transcription of the human placental *secreted alkaline phosphatase* (SEAP) as reporter. With this system, we screened 30 candidate *A. thaliana* proteins for light-dependent interactions with PhyA in mammalian cell culture (Fig. 2a and S1). CHO-K1 cells were transfected with these constructs and incubated for 24 h in darkness before supplementation with phycocyanobilin. For the remainder of the experiments, the cells were either kept in darkness for an additional 24 h or illuminated with 660 nm light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h. Several of the candidate interactors showed low SEAP reporter signals, indicating that they did not strongly interact with PhyA. Although other interactors exhibited higher SEAP readings, indicative of interactions with PhyA, most of them also showed high leakiness in the dark. However, one of the tested proteins, AT1G48770, showed a strongly pronounced light-dependent interaction with PhyA. The difference between basal and light-activated gene expression for the PPI pair formed by PhyA and this candidate protein amounted to 17-fold and was therefore comparable to that for the canonical

PhyB system (Fig. 2a). While overall expression under red light was lower compared to the PhyB-PIF system, basal gene expression in the dark was reduced as well. Based on this finding, we analyzed if the candidate protein cross-reacts with PhyB. In a derivative split transcription factor system with PhyB substituting PhyA, AT1G48770 showed no interaction with PhyB, neither in the dark nor upon red-light stimulation (Fig.2b). Thus, the AT1G48770 protein interacts solely with PhyA, and we therefore label it ‘only phytochrome A binding’ protein (OPA).

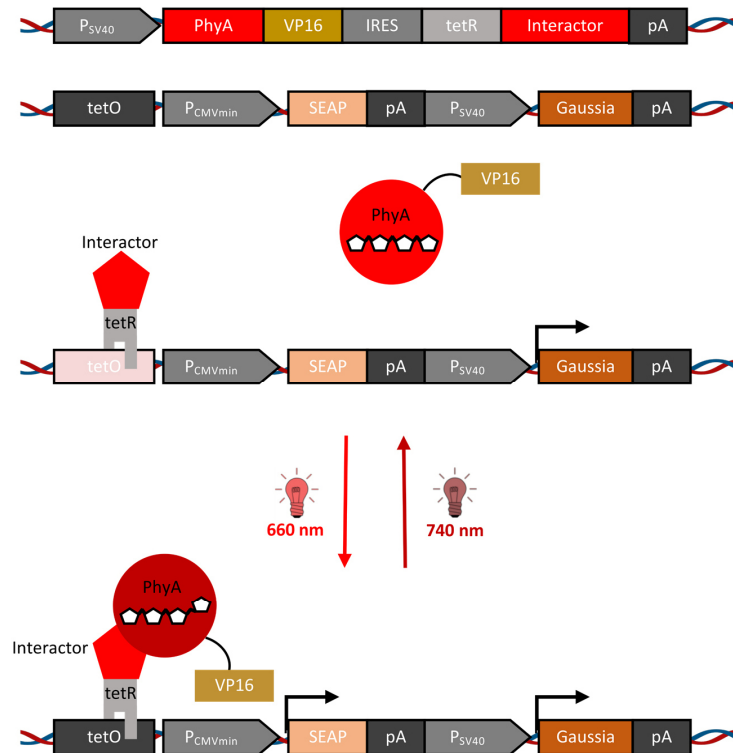


Figure 1: Design of the orthogonal red-light split transcription factor system. *Arabidopsis thaliana* phytochrome A (PhyA) is C-terminally fused to a virus-derived transactivator domain VP16 and separated by an IRES sequence from the potential interactors fused N-terminally to the tetracycline repressor domain (tetR). On the reporter cassette, 13 repeats of tetO are upstream of a synthetic CMV minimal promoter followed by the gene of human placental SEAP. On a second ORF, *Gaussia* Luciferase is under the control of the constitutive SV40 promoter as a normalization element. Upon red-light illumination, PhyA transitions into its active Pfr form which allows interaction with partner proteins. Therefore, VP16 is recruited to the minimal CMV promoter and induces expression of SEAP provided the interactor under study interacts with PhyA. Interaction and therefore transcription can be actively disrupted by illuminating with far-red light.

As mentioned above, the PhyB-PIF system can be realized with truncated interaction partners. To assess whether similar properties hold for the novel PhyA:OPA PPI pair, we tested shortened PhyA and OPA variants for light-dependent interaction. As PhyB only requires the NTE and the PSM for light-dependent PIF interactions (*Matsushita et al., 2003*), we analyzed if this also applies for the PhyA:OPA pair. We thus truncated PhyA directly after its PSM (amino acids 1-617). However, the

C-terminally shortened PhyA variant failed to activate SEAP gene expression, indicating a loss of function (Fig.3a). This suggests that OPA predominantly interact with the C-terminal OPM of PhyA rather than the PSM. We also shortened OPA to reduce the size of the interaction pair. To this end, the sequence of OPA was analyzed and a domain of unknown function (DUF1639; amino acids 125-176) was identified at the very C-terminus of the protein. To determine if this domain is needed for interaction with PhyA, we split OPA into two parts and analyzed them. While the part containing the DUF1639 domain failed to induce gene expression, the N-terminus of OPA (amino acids 1-90) still initiated SEAP expression like the full-length OPA (Fig.3a). Thus, the DUF1639 domain is not needed for an interaction with PhyA but likely serves other biological functions. To test, if the loss of the DUF1639 also results in a loss of orthogonality, the interaction between PhyB and the N-terminus of OPA (amino acid 1-90) was tested. However, no interaction of PhyB and the shortened OPA(1-90) was observed (Fig.3b).

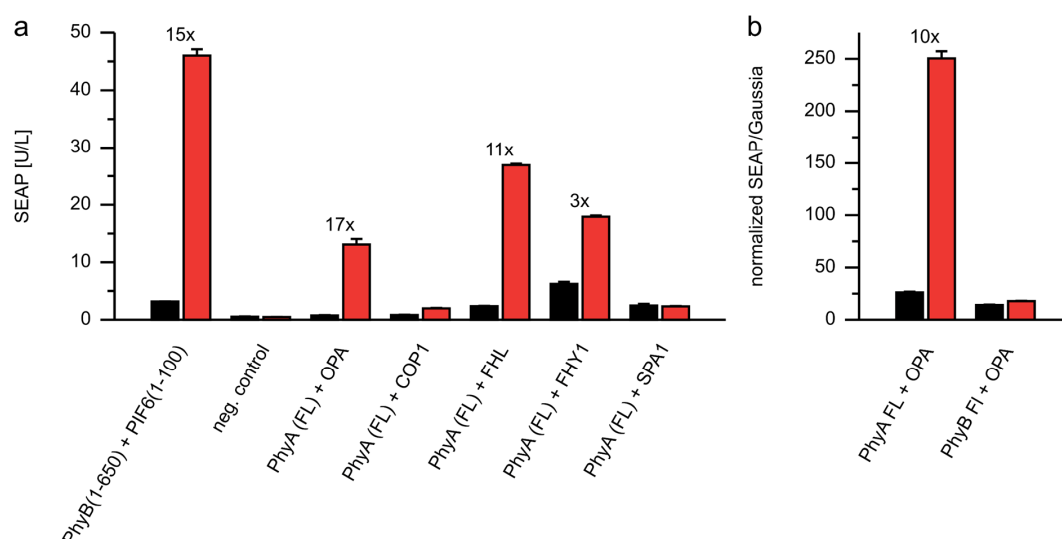


Figure 2: SEAP expression controlled by light-dependent protein-protein interaction. (a) Screening of candidate interactors of PhyA. CHO-K1 cells were transiently transfected with a reporter plasmid (pPF034) and the red-light split transcription factor system containing *Arabidopsis thaliana* phytochrome A (PhyA) (pPF007) and one of the candidate interactors. The published red-light split transcription factor system based on PhyB:PIF6 was also transfected as a control (Müller *et al.*, 2013a). Transfected cells were kept in dark for 24 h, supplemented with 15 μ M PCB and incubated for 1 h in darkness. Next, cells were illuminated for 24 h with 660-nm light at an intensity of 20 μ mol $\text{m}^{-2} \text{s}^{-1}$ (red bars) or kept in darkness (black bars). SEAP values were determined, and induction ratios calculated for 4 biological replicates. (b) OPA showed interaction with PhyA but not with PhyB. CHO-K1 cells were transfected with the reporter plasmid (pPF034) and plasmids containing PhyA or PhyB together with OPA to check for a light dependent interaction (pPF013; pPF078). Transfected cells were kept in darkness for 24 h, supplemented with 15 μ M PCB and incubated for 1 h in darkness. Next, cells were illuminated for 24 h with 660-nm light at an intensity of 20 μ mol $\text{m}^{-2} \text{s}^{-1}$ (red bars) or kept in darkness (black bars). SEAP and Gaussia values were determined, and SEAP was normalized with their Gaussia luciferase data. All experiments were done in 4 replicates, and the error bars indicate standard error of the mean.

To characterize the dependence on light dose of the PhyA-OPA(1-90) interaction, transfected cells were incubated under different red-light intensities. The system reached full induction after application of light doses as low as 2.5 μ mol $\text{m}^{-2} \text{s}^{-1}$ for 30 minutes (Fig.4a), which shows the high

sensitivity of the system. To evaluate the induction dynamics, we performed SEAP measurements as a function of time. Upon transfection of the PhyA-OPA(1-90) system, the CHO-K1 cells were illuminated for different periods with $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ red light, and SEAP values were determined. The SEAP expression under red light illumination rapidly ramped up over time while control cells kept in darkness displayed only poor expression levels (Fig.4b). A pronounced difference in expression between cells under red light and in the dark is observed as earlier as 4 hours after the onset of illumination, which again underlines the high sensitivity of the system. The expression was stable when cells were kept under red light for as long as 24 h. To assess the photoreversibility of the PhyA:OPA system, the experiment was performed under alternating cycles of red and far-red light in 24 h intervals (Fig.4c). When cells were illuminated with red light for 24 h, high levels of SEAP expression were detected. The contrary is observed if far-red light was applied instead of red light. After 24 h, the medium was exchanged, and illumination continued with either red or far-light. As in the initial stage of the experiment, the SEAP expression levels were solely dependent on the currently applied light conditions, and no memory effect of the prior illumination step was observed. Notably, the system showed a pronounced contrast between red and far-red light even after multiple cycles.

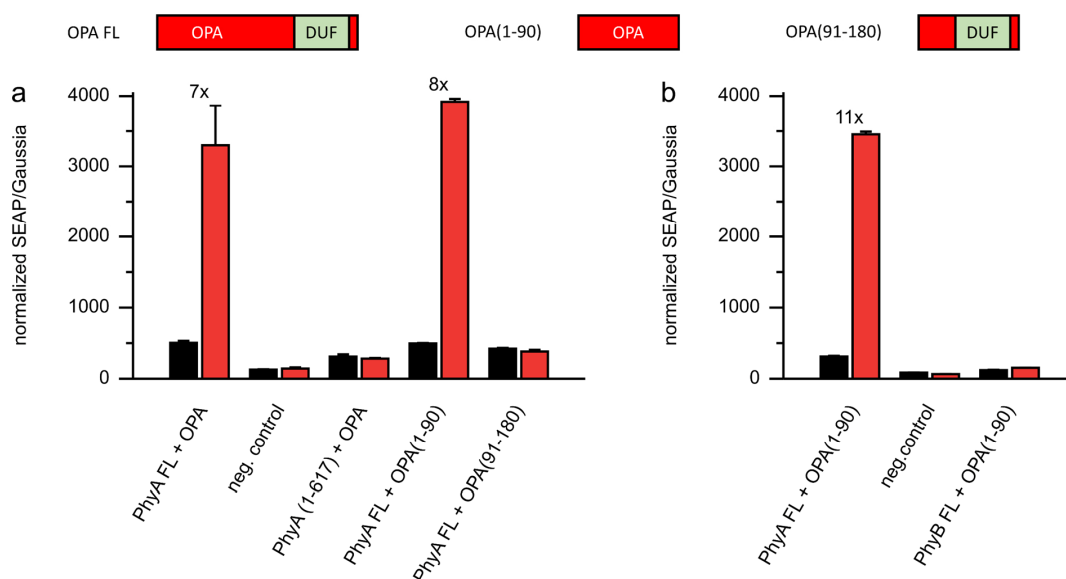


Figure 3: Optimization of the PhyA/OPA system. (a) Analysis of truncated PhyA and OPA variants. CHO-K1 cells were transfected with the reporter plasmid (pPF034) and the PhyA system as a control (pPF013) or the truncated versions of PhyA/OPA (pPF090; pPF086; pPF087). (b) Orthogonality of the PhyA:OPA system is independent of the DUF1679 domain. CHO-K1 cells were transfected with the reporter plasmid (pPF034) and the optimized PhyA system (pPF086) or PhyB FL combined with OPA 1-90 (pPF089). In (a) and (b) transfected cells were kept in darkness for 24 h, supplemented with $15 \mu\text{M}$ PCB and incubated for 1 h in darkness. Next, cells were illuminated for 24 h with 660-nm light at an intensity of $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (red bars) or kept in darkness (black bars). SEAP and Gaussia values were determined, and SEAP was normalized with their Gaussia luciferase data. All experiments were done in 4 biological replicates and the error bars indicate standard error of the mean. All plasmids were transfected in a molar ratio of 1:1 (w:w).

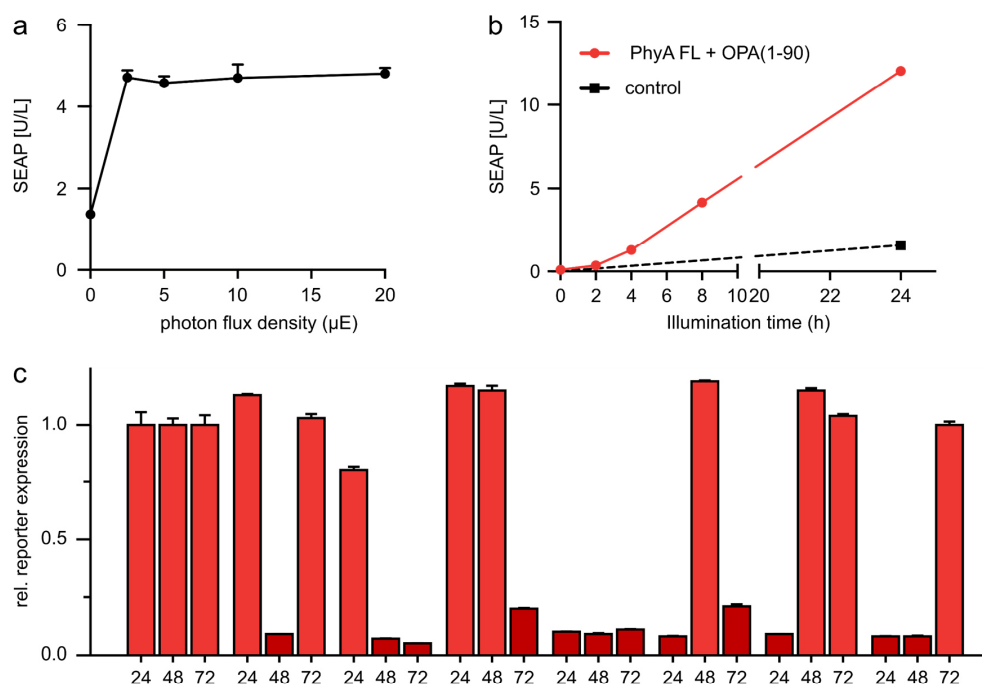


Figure 4: Characterization of the PhyA:OPA gene expression system. (a) Dose-response curve of the system. Transfected cells were illuminated with 660-nm light at intensities of 0, 2.5, 5, 10 and 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h. Afterwards, SEAP production was quantified. (b) Expression kinetics of the system. Transfected cells were illuminated with 660-nm light at an intensity of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. At the given timepoints (2 h, 4 h, 8 h, 24 h), SEAP levels were determined (red line). Control cells were kept in darkness for 24 h (black line). (c) Reversibility of the system. Transfected cells were supplemented with fresh medium containing 15 μM PCB after every 24 h and illuminated with the indicated wavelengths at an intensity of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Expression of SEAP was measured every 24 h and normalized by the values obtained for cells constantly illuminated with 660-nm light, to correct for changes in gene expression depending on growth over time. (a-c) CHO-K1 cells were transfected with the reporter plasmid (pPF034) and the optimized PhyA system (pPF086) at a molar ratio of 1:1 (w:w). Transfected cells were kept in darkness for 24 h, supplemented with 15 μM PCB and incubated for 1 h darkness, before being transferred to the indicated illumination conditions. All experiments were done in 4 biological replicates, and the error bars indicate standard errors of the mean.

While the split transcription factor system provides an efficient way to assess whether a PPI is occurring, it cannot provide quantitative information on the interaction strength of the PPI. To analyze the absolute affinity between PhyA and OPA and to compare it to the PhyB-PIF system, we applied fluorescence anisotropy measurements to record binding isotherms under red and far-red light (Golonka *et al.*, 2019). To this end, OPA and OPA(1-90) were fused to a C-terminal EYFP tag. If at a given condition PhyA and OPA are not interacting, the rotational diffusion and hence the fluorescence anisotropy of the EYFP tag should be unaffected. By contrast, if interaction between PhyA and OPA occurs, a complex of larger size and hydrodynamic radius is formed which reduces the rotational diffusion of the EYFP label and increases the fluorescence anisotropy. Based on this principle, we measured fluorescence anisotropy of a constant concentration amount of fluorescently labeled OPA protein and increasing concentrations of the PhyA. The dissociation

constant K_D was determined by fitting the data to a single-site binding isotherm. Doing so yielded an affinity of (450 ± 70) nM for the Pfr state interaction of full-length OPA and PhyA, while the Pr interaction was too weak for K_D to be exactly determined ($> 10 \mu\text{M}$) (Fig. 5a). The observed K_D values for both the Pfr- and Pr-state interaction of PhyA-OPA are in a similar range as those previously reported for the interaction between PhyB and PIF3/6 variants (*Golonka et al., 2019*). The fluorescence anisotropy measurements on OPA(1-90) and PhyA revealed weak interactions only, with a $K_D > 10 \mu\text{M}$ for the Pfr state and $> 20 \mu\text{M}$ for the Pfr state (Fig 5b). Although the interaction still depended on light, the affinity to PhyA is strongly decreased in OPA(1-90).

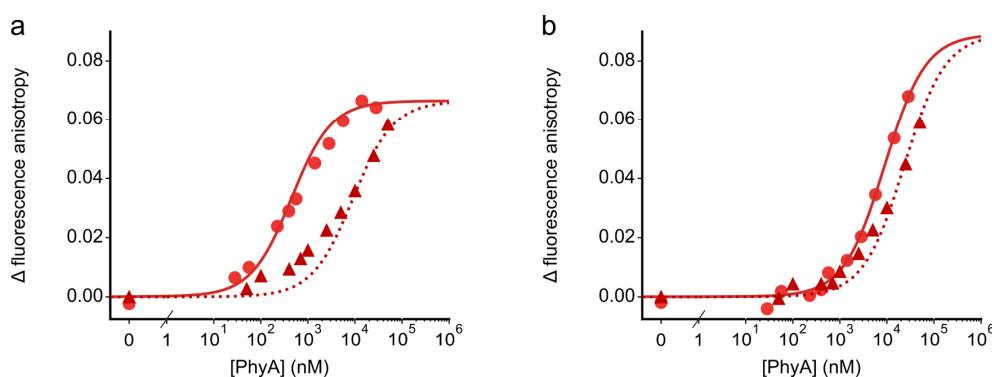


Figure 5: Quantitative analysis of light-dependent interaction between OPA-EYFP variants and PhyA by fluorescence anisotropy. (a) Fluorescence anisotropy measurements on 20 nM OPA-EYFP and increasing concentrations of PhyA after illumination with red light (circles) or far-red light (triangles). (b) Fluorescence anisotropy measurements on 20 nM OPA(1-90)-EYFP and increasing concentrations of PhyA after illumination with red light (circles) or far-red light (triangles).

Discussion

The red/far-red light-switchable bi-stable PhyB-PIF system is commonly used for gene expression control (*Müller et al., 2013a; Müller et al., 2014*), light induced protein localization (*Toettcher et al., 2011*) and for pathway control (*Toettcher et al., 2013*). PhyB-based systems offer the advantage over many other light-inducible systems of light-induced reversibility and deeper penetration into tissues (*Müller et al., 2014; Shimizu-Sato et al., 2002*). Although the system can be readily combined with other photoreceptor systems (*Müller et al., 2013b*), an orthogonal PPI pair that would allow the control of multiple cellular processes only with red light has not been reported. To fill this gap, we engineered a red-light-responsive split transcription factor system that is based on the interaction of PhyA with its newly identified interaction partner OPA. While crosstalk between full-length PIFs and different plant phytochromes has been reported (*Pham et al., 2018*), we did not observe any interaction between OPA and PhyB. The new red-light inducible optogenetic tool based on PhyA:OPA showed high sensitivity to red light and reversibility upon far-red illumination, thereby possessing very similar characteristics to the PhyB-PIF system. The interaction strength of OPA for

the Pfr and Pr states of PhyA is of the same magnitude as that for the PSM of PhyB and shortened PIF variants. We show that the photosensor module of PhyA on its own is not sufficient to enter PPIs with OPA. However, we could reduce the size of OPA to 90 amino acids, hence yielding OPA(1-90). While fluorescence anisotropy measurements reveal that the interaction strength of OPA(1-90) for both states of PhyA is strongly diminished, it still possesses a sufficiently large difference in affinity to allow light-dependent control of the transcription factor system.

We note that recently red-light dependent PPIs of a bacterial phytochrome and its natural binding partner were exploited to control gene expression in mammalian cell culture (*Redchuk et al., 2018*). In contrast to the plant phytochrome based systems, bacterial phytochromes utilize endogenous biliverdin and therefore supplementation with PCB is not needed. Although this bacterial phytochrome system should be orthogonal to those of plant phytochromes, it might not be best suited due to differences in spectral properties. The absorption maxima of bacterial phytochromes are much more red-shifted as those of plant phytochromes (*Kaberniuk et al., 2016*). On the one hand, red light used to activate plant phytochromes might activate bacterial phytochromes only weakly. On the other hand, far-red light triggers both the inactivation of plant phytochromes and depending on the exact wavelength applied either activation or inactivation of the bacterial system. Therefore, the precise control of both systems might be rather challenging. By contrast, PhyA exhibits the same spectral properties as PhyB, thereby activation and inactivation with red and far-red light, respectively, is straightforward.

Taken together, we introduce a novel PhyA-interactor OPA that functions in a light-dependent way similar to the already existing PhyB-PIF system, while being fully orthogonal to it. The PhyA system is a further addition to the optogenetic toolbox and can be applied for multi-pathway control via one wavelength.

Materials and methods

Plasmid construction

The design and construction of the expression vectors are described in table S1.

Cell culture, transfection, light induction

Chinese ovary hamster cells (CHO-K1; DSMZ, Braunschweig, Germany) were cultivated in HAMs D12 medium (PAN Biotech, Aidenbach, Germany; no.) supplemented with 10% (v/v) tetracycline-free fetal bovine serum (FBS; PAN Biotech; no. P30-3602; batch no. P080317TC) and 1.4% (v/v) streptomycin (PAN Biotech; no. P06-07100). Human embryonic kidney cells (HEK-293T; DSMZ, Braunschweig, Germany) and HeLa cells (HeLa; DSMZ, Braunschweig, Germany) were cultivated

in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech, Aidenbach, Germany; no. P04-03550) supplemented with 10% (v/v) tetracycline-free fetal bovine serum (FBS; PAN Biotech; no. P30-3602; batch no. P080317TC) and 1.4% (v/v) streptomycin (PAN Biotech; no. P06-07100). $5 \cdot 10^4$ CHO-K1, HEK-293T or HeLa cells were transfected using polyethylenimine (PEI; Polysciences Inc. Europe, Hirschberg, Germany; no. 23966-1) as in ³. 0.75 μ g DNA were diluted in 50 μ L OptiMEM (Invitrogen, Thermo Fisher Scientific) and mixed with a PEI/OptiMEM mix (2.5 μ L PEI solution in 50 μ L OptiMEM). The DNA-PEI mix was added to the cells after 15 min of incubation at room temperature. 4 h post transfection the medium was exchanged. If not indicated otherwise, all plasmids were transfected with PhyA system together with the reporter plasmid (pPF086; pPF034 in equal amounts (w/w). 24 h post transfection the cells were illuminated with 660-nm light for 24 h with a light intensity of 20 μ mol m⁻² s⁻¹ or kept in darkness. If not indicated otherwise, experiments were done in 4 biological replicates. For illumination, custom-built LED light-boxes with LED-panels emitting 660 nm were used (*Baaske et al., 2019*). Exchange of media and other cell-handling was done under 522-nm, safety light, to prevent inadvertent actuation of the light-sensitive systems.

SEAP assay

The supernatant of transfected cells was transferred to 96-well round-bottom plates and to inactivate endogenous phosphatases incubated at 68°C for 1 h. Afterwards, 80 μ L of the supernatant were transferred to a 96-well flat-bottom plates, and 100 μ L SEAP buffer (20 mM homoarginine, 1 mM MgCl₂, 21% (v/v) diethanolamine) was added per well (*Müller et al., 2013a*). After addition of 20 μ L 120 mM para-nitrophenyl phosphate, the absorption at 405 nm was measured for 1 h using a BMG Labtech CLARIOstar or a TriStar2 S LB 942 multimode plate reader (Berthold Technologies, Bad Wildbad, Germany). Outlier were statistically determined and excluded as described in (*Jacobs and Dinman, 2004*).

Gaussia luciferase assay

20 μ L of the supernatant of the transfected cells were transferred to a 96-well white plate and diluted in 60 μ L phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH₂PO₄, 8.03 mM Na₂PO₄, 137 mM NaCl). After addition of 20 μ L coelenterazine (472mM stock solution in methanol, diluted 1:1,500 in PBS; Carl Roth, Karlsruhe, Germany, no. 4094.4), the luminescence was measured for 20 min using a BMG Labtech CLARIOstar or a TriStar2 S LB 942 multimode plate reader (Berthold Technologies, Bad Wildbad, Germany).

Protein Purification

For PhyA expression, *E. coli* BL21(DE3) strain transformed with plasmid pDGxxx were grown in terrific-broth (TB) medium, supplemented with 100 $\mu\text{g mL}^{-1}$ streptomycin, at 37 °C in darkness. At an optical density at 600 nm (OD_{600}) of 0.7 – 0.9, δ -Aminolevulinic acid was supplemented at 0.5 mM to assist chromophore production (*Gambetta and Lagarias, 2001*), and the expression was induced by adding 1 mM β -D-1-thiogalactopyranoside (IPTG). The temperature was decreased to 18 °C and cultivation continued overnight. Cells were harvested by centrifugation, resuspended in lysis buffer [50 mM Tris/HCl pH 8.0, 20 mM NaCl, 20 mM imidazole; supplemented with protease inhibitor mix (cOmplete Ultra, RocheDiagnostics, Mannheim, Germany)]. Lysis was done by sonification and cell fragments were removed by centrifugation. Supernatant was applied to a Protino Ni-NTA 1 mL columns (Macherey-Nagel, Düren, Germany) and eluted with a linear imidazole gradient from 20 to 500 mM. Elution fractions were analyzed by sodiumdodecylsulfat polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 1 mM Zn^{2+} to enable detection of covalently incorporated bilin chromophores via zinc-induced fluorescence (*Berkelman and Lagarias, 1986*). Suitable fractions were pooled and dialyzed overnight into AEX buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl, 5 mM 2-mercaptoethanol). Anion exchange chromatography was done on a HiTrap Q HP 1 mL column (GE Healthcare EuropeGmbH, Freiburg, Germany). Proteins were eluted using two successive linear gradients from 50 to 300 mM NaCl and from 300 to 500 mM. Elution fractions were analyzed by SDS-PAGE, pooled and dialyzed against storage buffer [10 mM Tris/HCl pH8, 10 mM NaCl, 10 % (w/v) glycerol] before stored at –80 °C.

Purification of OPA/OPA(1-90)-EYFP was done with a similar protocol with the following differences. Cells were cultivated in lysogenic broth (LB) instead of TB. No δ -aminolevulinic acid was added, and incubation after induction continued at 16 °C overnight. Following IMAC, the N-terminal His6-SUMO was cleaved overnight at 4 °C during dialysis into 50 mM Tris/HCl pH 8.0 and 20 mM NaCl using SENP2-protease. A second IMAC was done to remove cleaved His6-SUMO, and the flow-through containing the OPA construct was collected. If needed, OPAs were further purified by AEX chromatography as described above. Finally, OPA variants were dialyzed against storage buffer and stored at –80 °C.

Fluorescence anisotropy

Phytochrome samples were illuminated with either (640 ± 15) nm ($65 \mu\text{W cm}^{-2}$) or (750 ± 15) nm ($420 \mu\text{W cm}^{-2}$) before the experiment. 20 nM OPA-EYFP or OPA(1-90)-EYFP and increasing concentrations of PhyA were prepared in 20 mM HEPES/HCl pH 7.3, 10 mM NaCl and 100 $\mu\text{g mL}^{-1}$ bovine serum albumin. The mixtures were transferred into black 384-well plates (Brand, Wertheim, Germany) and illuminated with either 640 nm or 750 nm prior to the measurement in a CLARIOstar

MTP reader (BMG Labtech). The extinction and detection wavelength were 482 ± 16 nm and 530 ± 40 nm, respectively, and a 504 nm long-pass dichroic filter was used. The fluorescence anisotropy value was adjusted to 0.315 previously recorded for EYFP (*Golonka et al., 2019*). Anisotropy data was fitted to equation (1) using the Fit-o-mat software (*Möglich, 2018*):

$$r = r_0 + \frac{r_1}{2} \times \left\{ 1 + \frac{[\text{PhyA}]}{c_{\text{tot}}} + \frac{K_D}{c_{\text{tot}}} - \sqrt{\left(1 + \frac{[\text{PhyA}]}{c_{\text{tot}}} + \frac{K_D}{c_{\text{tot}}} \right)^2 / 4 - \frac{[\text{PhyA}]}{c_{\text{tot}}}} \right\} \quad (1)$$

where r represents the anisotropy of either OPA-EYFP or OPA(1-90)-EYFP, $[\text{PhyA}]$ is the concentration of phytochrome in the Pr or Pfr state, $c_{\text{tot}} = 20$ nM is the concentration of either OPA-EYFP or OPA(1-90)-EYFP, and K_D is the dissociation constant.

Statistical analysis

All data analysis was performed using Microsoft Excel, GraphPad Prism 6 or the Fit-o-mat software (*Möglich, 2018*). Statistical outlier was determined and excluded as described in (*Jacobs and Dinman, 2004*).

Author contributions

PF, DG, SF planned and performed experiments. PF supervised SF and analyzed the data of cell culture experiments. DG purified proteins, furthermore, performed and evaluated fluorescence anisotropy measurements. AM, AH and MDZ conceived, designed and supervised the research, and contributed to analyzing the experiments. PF and DG prepared the figures. PF, DG, AM and MDZ wrote the manuscript.

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Supplemental

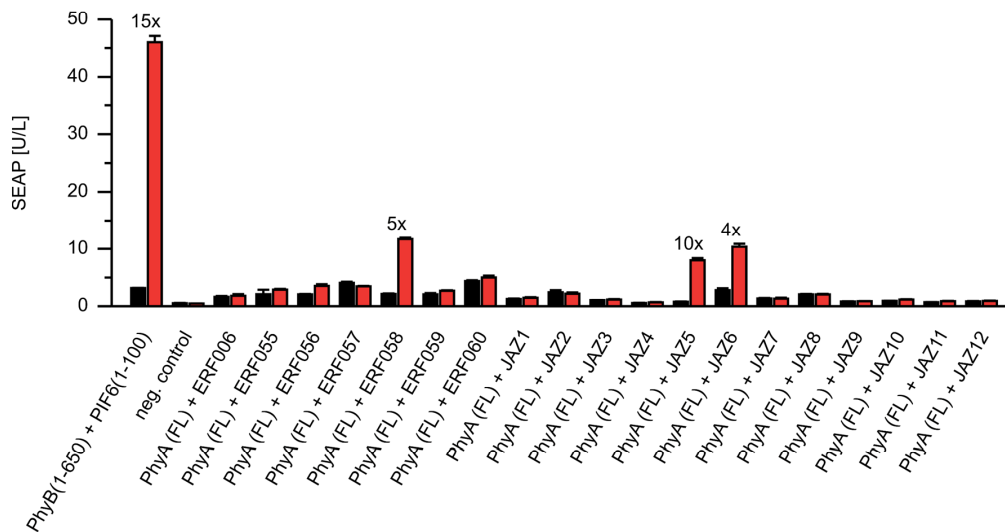


Figure 6: PhyA potential interactor screening. Screening of potential interactors of PhyA. CHO-K1 cells were transiently transfected with the described reporter plasmid (pPF034) and the plasmids based on the red-light split transcription factor system containing *Arabidopsis thaliana* phytochrome A (PhyA) (pPF007) and one of the potential interactors. Additionally, the published red-light split transcription factor system was also transfected as a control. Transfected cells were kept in dark for 24 h, supplemented and incubated for 1 h with 15 μ M PCB and illuminated afterwards for 24 h with 660 nm and an intensity of 20 μ mol m⁻² s⁻¹ (red bars) or kept in dark (black bars). SEAP values were determined, and induction folds calculated of 4 replicates. All experiments were done in 4 replicates and the error bars indicate standard deviation of the mean. All plasmid were transfected in 1:1 molar ratio (w:w)

Supplementary Table S1: Plasmids designed and used in this study.

All plasmids were cloned using AQUA (source) or Gibson Cloning (source)

Plasmid	Description	Reference or source
pKM022	P_{SV40}-PhyB¹⁻⁶⁵⁰-VP16-NLS-IRES-tetR-PIF6¹⁻¹⁰⁰-pA	
pPF001	P_{SV40}-SpeI-PhyB¹⁻⁶⁵⁰-EcoRV-VP16-NLS-IRES-tetR-BsrGI-PIF6¹⁻¹⁰⁰-Ascl-pA pKM022 was amplified using oligos oPF001/oPF002 to add <i>SpeI</i> and <i>Ascl</i> restriction site. PhyB ¹⁻⁶⁵⁰ was amplified from pKM022 with oPF003/oPF004 to add <i>EcoRV</i> restriction site. PIF6 ¹⁻¹⁰⁰ was amplified from pKM022 with oPF005/oPF006 to add <i>BsrGI</i> restriction site. VP16-NLS-IRES-tetR was excised by <i>EcoRV</i> from pMZ1200. All fragments were assembled by AQUA cloning.	This work
pKM006	tetO₁₃-P_{CMVmin}-SEAP-pA	
pPF034	tetO₁₃-P_{CMVmin}-SEAP-pA-P_{SV40}-Gaussia-pA pKM006 was linearized using <i>HindIII</i> and <i>SpeI</i> . BGH-pA was amplified using oligos oPF007/oPF008 from pKM528. P _{SV40} was amplified using oligos oPF009/oPF010 from pKM022. Gaussia was	This work

	amplified using oligos oPF047/oPF048 from pKM083. All fragments were assembled by AQUA cloning.	
pPF007	P_{SV40}-SpeI-PhyA-EcoRV-VP16-NLS-IRES-tetR-BsrGI-PIF6¹⁻¹⁰⁰-Ascl-pA pPF001 was linearized by <i>SpeI</i> and <i>EcoRV</i> . PhyA was amplified from pMZ1160 by using oligos oPF025/oPF026. Both fragments were assembled by AQUA.	This work
pPF013	P_{SV40}-SpeI-PhyA-EcoRV-VP16-NLS-IRES-tetR-BsrGI-AAB2-Ascl-pA pPF007 was linearized by <i>Ascl</i> and <i>BsrGI</i> . AAB2 was amplified using oligos oPF100/oPF101. Both fragments were assembled by AQUA.	This work
pPF086	P_{SV40}-SpeI-PhyA-EcoRV-VP16-NLS-IRES-tetR-BsrGI-AAB2¹⁻⁹⁰-Ascl-pA pPF007 was linearized by <i>BsrGI</i> and <i>Ascl</i> . AAB2 ¹⁻⁹⁰ was amplified using oligos oPF353/oPF101 from pPF013. Both fragments were assembled by AQUA.	This work
pPF087	P_{SV40}-SpeI-PhyA-EcoRV-VP16-NLS-IRES-tetR-BsrGI-AAB2⁹¹⁻¹⁸⁰-Ascl-pA pPF007 was linearized by <i>BsrGI</i> and <i>Ascl</i> . AAB2 ⁹¹⁻¹⁸⁰ was amplified using oligos oPF354/oPF100 from pPF013. Both fragments were assembled by AQUA.	This work
pPF078	P_{SV40}-SpeI-PhyB-EcoRV-VP16-NLS-IRES-tetR-BsrGI-AAB2-Ascl-pA pPF009 was linearized by <i>AsiSI</i> and <i>Ascl</i> . AAB2 was amplified using oligos oPF339/oPF340. Both fragments were assembled by AQUA.	This work
pPF090	P_{SV40}-SpeI-PhyA¹⁻⁶¹⁷-EcoRV-VP16-NLS-IRES-tetR-BsrGI-AAB2-Ascl-pA pPF013 was amplified using oligos oPF360/oPF361 and were assembled by AQUA.	This work
pPF089	P_{SV40}-SpeI-PhyB-EcoRV-VP16-NLS-IRES-tetR-BsrGI-AAB2¹⁻⁹⁰-Ascl-pA pPF009 was linearized by <i>AsiSI</i> and <i>Ascl</i> . AAB2 ¹⁻⁹⁰ was amplified using oligos oPF353/oPF359 from pPF013. Both fragments were assembled by AQUA.	This work
pDG321	pCDFduet1-PhyA-His6	This work
pDG452	pET19b-His6-SUMO-OPA-EYFP	This work
pLH058	pET19b-His6-SUMO-OPA(1-90)-EYFP	This work

5.5 Manuskript V

Titel: „Illuminating a phytochrome paradigm – a light-activated phosphatase in two-component signaling uncovered”

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Illuminating a Phytochrome Paradigm – a Light-Activated Phosphatase in Two-Component Signaling Uncovered

Elina Multamäki¹, Rahul Nanekar², Dmitry Morozov³, Topias Lievonen², David Golonka⁴, Weixiao Yuan Wahlgren⁵, Brigitte Stucki-Buchli², Jari Rossi¹, Vesa P. Hytönen⁶, Sebastian Westenhoff⁵, Janne A. Ihalainen^{2,*}, Andreas Möglich⁴, Heikki Takala^{1,2,*}

¹ Faculty of Medicine, Anatomy, University of Helsinki, 00014 Helsinki, Finland

² Nanoscience Center, Department of Biological and Environmental Sciences, University of Jyväskylä, 40014 Jyväskylä, Finland

³ Nanoscience Center, Department of Chemistry, University of Jyväskylä, 40014 Jyväskylä, Finland

⁴ Lehrstuhl für Biochemie, Universität Bayreuth, 95447 Bayreuth, Germany

⁵ University of Gothenburg, Department of Chemistry and Molecular Biology, 40530 Gothenburg, Sweden

⁶ Faculty of Medicine and Health Technology, BioMediTech, Tampere University, 33520 Tampere, Finland; Fimlab Laboratories, 33520 Tampere, Finland

*To whom correspondence should be addressed: Heikki Takala: Faculty of Medicine, University of Helsinki, FI-00014 Helsinki, Finland; E-mail: heikki.takala@helsinki.fi; Tel.: (+358) 2941-25238. Janne Ihalainen: Department of Biological and Environmental Sciences, University of Jyväskylä, FI-40014, Jyväskylä; E-mail: janne.ihalainen@jyu.fi; Tel.: (+358) 400-247979.

Keywords: phosphatase, phytochrome, response regulator, sensory photoreceptor, structural biology, two-component signaling

ABSTRACT

Bacterial phytochrome photoreceptors usually belong to two-component signaling systems which transmit environmental stimuli to a response regulator through a histidine kinase domain. Phytochromes switch between red light-absorbing and far-red light-absorbing states. Despite exhibiting extensive structural responses during this transition, the model bacteriophytochrome from *Deinococcus radiodurans* (DrBphP) lacks detectable kinase activity. Here, we resolve this long-standing conundrum by comparatively analyzing the interactions and output activities of DrBphP and a bacteriophytochrome from *Agrobacterium fabrum* (AgP1). Whereas AgP1 acts as a conventional histidine kinase, we identify DrBphP as a light-sensitive phosphatase. While AgP1 binds its cognate response regulator only transiently, DrBphP does so strongly, which is rationalized at the structural level. Our data pinpoint two key residues affecting the balance between kinase and phosphatase activities, which immediately bears on photoreception and two-component signaling. The opposing output activities in two highly similar bacteriophytochromes inform the use of light-controllable histidine kinases and phosphatases for optogenetics.

INTRODUCTION

Two-component signaling systems are mainly found in prokaryotes and allow cells to respond to various environmental signals (Stock et al., 2000). These systems have been under extensive research ever since their discovery, as they control a wide range of cellular mechanisms from enzymatic activity to transcription regulation (Stock et al., 1989). A canonical two-component system consists of a homodimeric sensor histidine kinase (HK) and its cognate response regulator (RR) (West and Stock, 2001). To the extent it has been studied, most HK proteins sense chemical signals and generally reside within the plasma membrane (Stock et al., 1989). The output activity is exerted by an intracellular HK module, consisting of two subdomains: a dimerization histidine phosphotransfer (DHP) domain, and a catalytic ATP-binding (CA) domain. Based on their DHP sequence, the HK proteins can be divided into four subtypes that are called HisKA, HisKA_2, HWE_HK and HisKA_3 (El-Gebali et al., 2018; Jacob-Dubuisson et al., 2018).

The HK catalyzes autophosphorylation and subsequent phosphotransfer to the cognate RR. During the autophosphorylation reaction, the eponymous histidine of the DHP domain is phosphorylated (Bijlsma and Groisman, 2003; Marina et al., 2001), either within the same monomer (*cis*) or the sister molecule of the homodimer (*trans*) (Buschiazzi and Trajtenberg, 2019). In the phosphotransfer reaction, the phosphate is relayed to a conserved aspartate residue within a receiver (REC) domain of the RR. This reaction entails RR activation and elicits output responses such as altered gene expression (Bijlsma and Groisman, 2003; Casino et al., 2009; West and Stock, 2001).

HKs may also act as phosphatases that hydrolyze the phospho-aspartyl bond in the phosphorylated response regulator, thus resetting the two-component system (Goulian, 2010; Russo and Silhavy, 1993). Whereas the kinase activity has been extensively studied (Casino et al., 2014), the importance of the phosphatase activity has been appreciated more recently (Huynh et al., 2011; Kostakioti et al., 2009; Willett and Kirby, 2012). In two-component systems, a dynamic balance between kinase and phosphatase activities exists that determines the net output and downstream physiological effects. Different conformational states of the HK are necessary for balancing these activities (Gao and Stock, 2013; Trajtenberg et al., 2016).

In contrast to the transmembrane receptors that constitute the vast majority of all HKs, receptors sensitive to light are frequently soluble, which greatly benefits their structural and mechanistic analyses (Diensthuber et al., 2013; Jacob-Dubuisson et al., 2018; Möglich, 2019). As a case in point, phytochromes are red/far-red light-sensing photoreceptors that regulate diverse physiological processes in plants, fungi, and bacteria (Quail et al., 1995), e.g., chromatic adaptation and phototaxis in prokaryotes (Rockwell et al., 2006). Bacterial phytochromes (BphPs) usually belong to two-component signaling systems, with a cognate response regulator commonly encoded in the same operon (Arnold et al., 2006; Bhoo et al., 2001; Möglich, 2019). BphPs comprise an N-terminal photosensory module (PSM) with PAS (Period/ARNT/Single-minded), GAF (cGMP phosphodiesterase/adenylyl cyclase/FhlA) and PHY (Phytochrome-specific) domains (Wagner et al., 2007). The PSM binds a biliverdin IX α chromophore via a thioether linkage to its conserved cysteine

within the PAS domain (Burgie et al., 2014; Lamparter, 2004). The PSM is followed by a C-terminal output module, which is usually a HK domain.

Photoactivation by red and far-red light drives biliverdin *Z/E* isomerization, which underlies the phytochrome switch between its red light-absorbing (Pr) and far-red light-absorbing (Pfr) states (Esteban et al., 2005). In the absence of light, phytochromes revert to their dark-adapted resting state thermally in a process called dark reversion. Whereas the Pr state is the dark-adapted and most stable state in canonical phytochromes, it is the Pfr state in bathy phytochromes (Karniol and Vierstra, 2003). As first demonstrated for the model bacteriophytochrome from *Deinococcus radiodurans* (DrBphP), light induces extensive structural changes in the photosensory module that are relayed to the output module (Takala et al., 2014a).

The enzymatic activity of several phytochrome-HKs has been investigated as a function of light environment. In the cyanobacterial phytochrome Cph1, the dark-adapted Pr state exhibited higher kinase activity than the Pfr state (Esteban et al., 2005; Hübschmann et al., 2001; Yeh et al., 1997), as is the case for other bacteriophytochromes (Giraud et al., 2005). In particular, the bacteriophytochrome from *Agrobacterium fabrum* (AgP1) displays histidine kinase activity in its resting Pr state (Karniol and Vierstra, 2003; Lamparter et al., 2002); in the Pfr state, the autophosphorylation and phosphotransfer reactions are down-regulated by 2-fold and 10-fold, respectively (Karniol and Vierstra, 2003). The light-activated kinase activity of the AgP1 has been shown to control bacterial conjugation (Bai et al., 2016). By contrast, no kinase activity has been demonstrated for DrBphP, notwithstanding close sequence homology and the elaborate structural changes this receptor undergoes under light (Bhoo et al., 2001; Takala et al., 2014a). Despite the eminent role of DrBphP as a paradigm for photoreception, the enzymatic activity and the physiological role of this model phytochrome have hence remained enigmatic.

Here, we unravel this long-standing puzzle by studying in detail the enzymatic activity and interactions of DrBphP and AgP1, as two canonical bacteriophytochromes with HK effector domains. By pursuing an integrated biochemical and structural strategy, we show that despite close homology, AgP1 acts as a histidine kinase whereas DrBphP can be assigned as a light-activated phosphatase. Our biochemical and structural data pinpoint two key residues proximal to the catalytic histidine that affect the balance between the kinase and phosphatase activities. Together, the two phytochromes provide soluble, light-controllable systems with opposite activities for the study and application of two-component signaling.

RESULTS

The spectral properties of DrBphP are affected by DrRR

We investigated by UV-vis absorption spectroscopy, whether the presence of the response regulators affects the photocycle of the bacteriophytochromes DrBphP and AgP1. For comparison, we generated a hybrid receptor, denoted ‘Chimera’ in the following, which comprises the DrBphP PSM and the AgP1 HK domain (Figure 1A). First, we assessed whether the Pr- and Pfr-state absorption spectra are influenced by RR addition. DrBphP, AgP1 and Chimera all showed typical absorption spectra with Soret and Q-band absorption peaks for both states, which were unaffected by the addition of the cognate RR (Figure 1B). We then analyzed the dark reversion of phytochrome samples after saturating red light (655 nm), with and without excess RR. In all cases, the recovery exhibited multiple exponential phases (Supporting Figure S1A). The recovery of AgP1 was notably faster than that of DrBphP, but the rate of dark reversion in the Chimera was between that of DrBphP and AgP1. Earlier studies indicate that the dark reversion in phytochromes is affected by the dimerization interfaces in both the PSM and HK domain (Takala et al., 2015). In line with this notion, our results indicate that the spectral characteristics of the Chimera are governed by both the AgP1 HK and DrBphP PSM.

The dark reversion kinetics of AgP1 and Chimera were not particularly affected by the response regulator from *Agrobacterium fabrum* (AtRR), but the dark reversion of DrBphP was significantly accelerated by *Deinococcus radiodurans* response regulator (DrRR). Although the underlying molecular mechanism is unclear, this finding indicates that DrRR binds to the DrBphP HK and favors adoption of the Pr state. Interestingly, this observation is opposite to that for *Arabidopsis thaliana* phytochrome B, where the phytochrome-interacting factor (PIF) bound to and stabilized the Pfr state (Golonka et al., 2019).

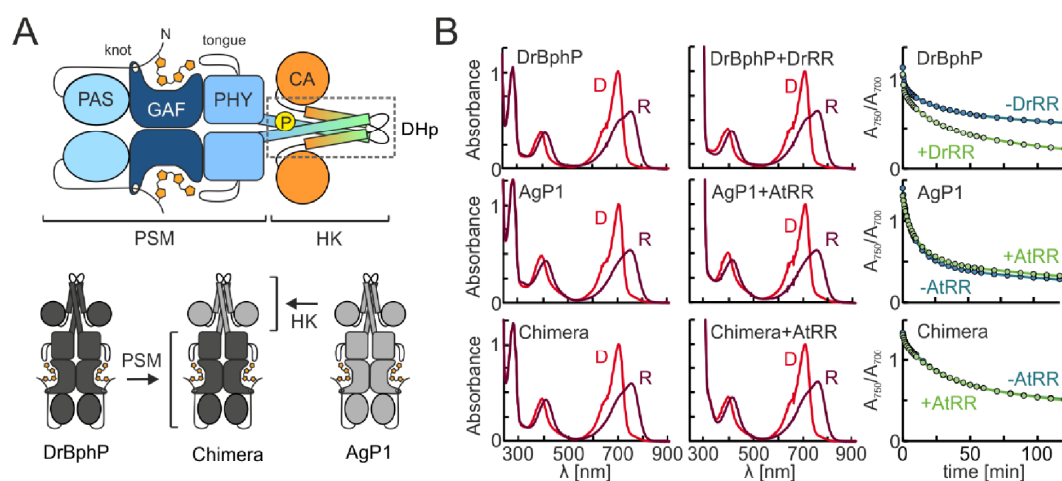


FIGURE 1. Overall structure and UV-vis spectroscopy of DrBphP, AgP1, and their chimera samples with and without their cognate response regulator. **A.** Schematic representation of a canonical bacteriophytochrome with an effector histidine kinase (HK) domain. The site of phosphorylated histidine is indicated as letter ‘P’. In addition, a schematic

presentation of the phytochrome chimera is shown, where the photosensory module (PSM) of DrBphP is combined with the HK domain of AgP1. Domain abbreviations: Period/ARNT/Single-minded (PAS), cGMP phosphodiesterase/adenylyl cyclase/FhlA (GAF), Phytochrome-specific (PHY), Histidine kinase (HK), Dimerization Histidine phosphotransfer domain (DHP), catalytic ATP-binding domain (CA). **B.** The absorption spectra of the BphP HKs with and without RR in dark (D) or under red light (R), and their dark reversion kinetics. The dark reversion is shown as an A_{750}/A_{700} ratio over time, where 0 min corresponds to the time the 655 nm illumination ceased. The dark reversion rates of AgP1 and Chimera were unaffected by AtRR, but DrBphP reversion is facilitated by DrRR.

DrBphP interacts with DrRR more strongly than AgP1 with AtRR

To further analyze the interaction between phytochromes and their RRs, we applied size-exclusion chromatography and fluorescently labeled RR proteins (Supporting Figure S1B). We analyzed the BphP and RR proteins individually or as pairs after saturating far-red or red illumination. The addition of DrBphP to the sample caused a shift in the retention of EGFP-labeled DrRR, indicative of a binding interaction independent of illumination. By contrast, the EGFP-AtRR retention was not significantly affected by the presence of AgP1, therefore arguing against a stable interaction between AgP1 and AtRR (Supporting Figure S1).

To investigate the BphP/RR interactions in more detail with minimal measurement delay and dark reversion after light exposure, we resorted to surface plasmon resonance (SPR). The response regulators were coupled to SPR chips, and the changes in the SPR signal were measured while applying the phytochromes to the sensor surface. We first assessed binding of DrRR to DrBphP in the Pr state by evaluating the steady-state saturation signal (Figure 2A) and obtained a dissociation constant K_D of $(43 \pm 8) \mu\text{M}$ when assuming a 1:1 binding stoichiometry (Figure 2C). This value was verified by Langmuir kinetic analysis which yielded an affinity of comparable strength ($K_D \sim 10 \mu\text{M}$).

The interaction of the DrBphP/DrRR pair was little affected by illumination, with lower signal amplitude and a K_D value of $(60 \pm 7) \mu\text{M}$ upon red-light application. The interaction between AgP1 and AtRR was substantially weaker ($K_D > 200 \mu\text{M}$), and no saturation of the binding curve could be reached. The shape of the SPR response graph indicates that the association and dissociation kinetics of the AgP1/AtRR are fast, which precludes the kinetic evaluation of this interaction. That notwithstanding, the AgP1/AtRR interaction was unaffected by red light (Supporting Figure S2C).

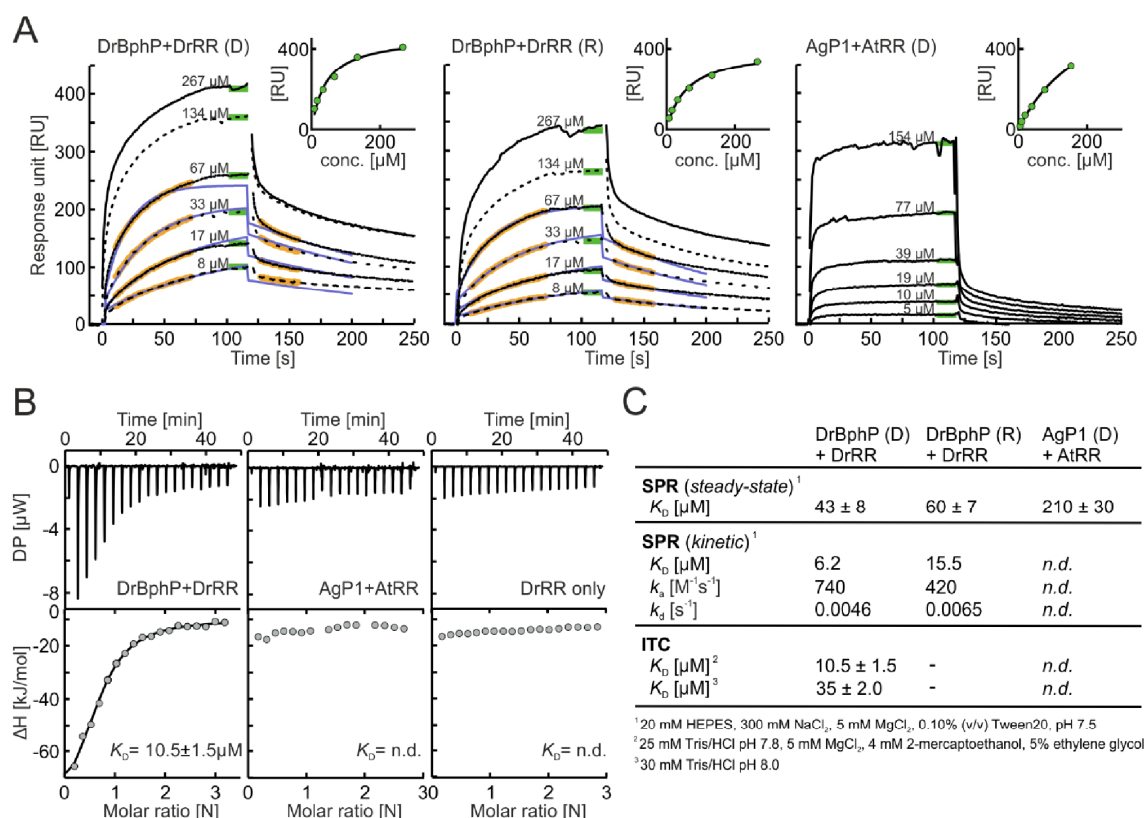


FIGURE 2. BphP/RR interactions. **A.** Surface plasmon resonance (SPR) measurements. The response regulators were coupled on the sensor surface, and varying concentrations of the corresponding phytochrome were applied on the sensor. The sensorgrams (black lines), the kinetic fits (blue lines) and the parts of the data that were used for kinetic analysis (orange) are indicated. For kinetic analyses, DrBphP concentrations of 8–67 μ M were used. Green lines mark the R_{eq} values that were used for evaluating the steady state affinity data, shown in panel **C**. Inset: Steady-state fit of the concentration series, where R_{eq} values were used for affinity approximation. DrBphP showed intermediate-affinity binding to DrRR-coupled surface in dark ($K_D = 43 \pm 8 \mu$ M), which was not notably affected by saturating red light ($K_D = 60 \pm 7 \mu$ M). Only weak interaction ($K_D > 200 \mu$ M) was detected between AgP1 and AtRR. **B.** Isothermal titration calorimetry (ITC) measurements. Differential power (DP) resulting from the injections is plotted against time, and binding enthalpy (ΔH) is plotted against the molar ratio of the proteins. **C.** Table summarizing the results from SPR and ITC analyses. See Supporting Figure S2D for a table with additional SPR fitting values and additional ITC measurements.

As a complementary method, we applied isothermal calorimetry (ITC) to obtain information on the phytochrome interactions in the dark. The DrBphP/DrRR interaction occurs in 1:1 stoichiometry with a K_D of $(10.5 \pm 1.5) \mu$ M (Figure 2B), slightly lower than the value attained by SPR (Figure 2C). Unlike in a blue light-regulated HK (Sharda et al., 2009), this interaction was not affected by the addition of ATP or its analogue AMP-PNP (Supporting Figure S2B). AgP1 binding to AtRR could not be detected by ITC (Figure 2B), consistent with the SEC data and the fleeting binding seen in SPR. The binding parameters were highly similar also in a different buffer condition (Figure 2C, Supporting Figure S2A).

Taken together, the interactions of DrBphP and AgP1 with their cognate response regulators were clearly different. Next we studied whether these differences in the interaction parameters correlate with enzymatic activity, as we speculate that the function of these phytochromes is reflected in their interactions.

AgP1 functions as a histidine kinase but DrBphP acts as a phosphatase

We characterized the kinase activity of DrBphP and AgP1 by ^{32}P - γ -ATP autoradiography (Figure 3A). The autophosphorylation reaction of AgP1 occurred preferably in the Pr state and is reduced after red light illumination, consistent with previous reports (Lamparter et al., 2002). If AtRR was present, it received a phosphate from AgP1 in the phosphotransfer reaction. This reaction occurred preferably in the dark-adapted Pr state but was almost absent under constant red-light illumination (i.e., in Pfr state). This verifies that AgP1 binds to and transfers its phosphate to AtRR in its active Pr state.

Intriguingly, DrBphP lacked autokinase or phosphotransfer activities both in the Pr and Pfr state (Figure 3A). The absence of kinase activity is surprising as the DrBphP and its PSM evidently undergo light-induced structural changes that seem to be conserved among other phytochromes (Bjorling et al., 2016; Bjorling et al., 2015; Takala et al., 2014a). Moreover, all homologous bacteriophytochrome HKs studied to date exhibited light-dependent kinase activity. We reasoned that the unusual absence of kinase activity in DrBphP could in principle be due to 1) lack of interaction with the DrRR; 2) inability of its PSM to transduce signals to the HK effector; or 3) inactivity of the DrBphP HK module. Scenario 1 can be ruled out right away, as the above measurements by three independent methods consistently showed interaction between DrBphP and DrRR. To discriminate between the scenarios 2 and 3, we assessed the histidine kinase activity of the Chimera and found that overall it functioned similarly to wild-type AgP1, in that it had robust autokinase and phosphotransfer activity in the Pr state, but much reduced activity in the Pfr state (Figure 3A). This result clearly states that DrBphP undergoes productive structural changes that are conducive to controlling HK activity, thereby ruling out scenario 2

To address scenario 3, we continued the analysis by applying Phos-tag gels where unphosphorylated proteins and their phosphorylated counterparts can be resolved based on migration through the gel matrix. Although the phytochromes were not separated from their phosphorylated forms, unphosphorylated and phosphorylated response regulators were clearly resolved from another (Figure 3B). The assay confirmed that the wild-type AgP1 phosphorylates AtRR preferably in the Pr state and revealed that it is also able to cross-phosphorylate DrRR with similar efficiency (Figure 3B). However, like in the radiolabeling assay (Figure 3A), DrBphP lacked kinase activity as it could not produce phosphorylated DrRR (phospho-DrRR).

The residue that immediately follows the catalytic histidine, denoted as H+1, is acidic in the majority of sensor histidine kinases and has been implicated in the autophosphorylation reaction (Casino et al., 2014). Whereas AgP1 has Asp529 in this position and thus conforms to the prevalent sequence motif, DrBphP unusually possesses a histidine in the corresponding position 533 (Figure 3F). To test the role of the H+1 position, we generated the AgP1 D529H and DrBphP H533D variants. The D529H

mutation rendered AgP1 inactive (Figure 3B), verifying the importance of this acidic residue for the kinase activity. Like the wild-type DrBphP, the H533D variant did not show any histidine kinase activity (Figure 3B and E). Therefore, this single mutation in the H+1 position is not sufficient to rescue the kinase activity of DrBphP.

As sensor histidine kinases may also function as phosphatases (Herrou et al., 2017; Möglich et al., 2009; Russo and Silhavy, 1993), we tested the DrBphP and AgP1 HKs in that regard. Of particular advantage, the Phos-tag gels allow to assess the dephosphorylation of phospho-RR proteins. To this end, we generated the phosphorylated response regulators chemically by treatment with acetyl phosphate (McCleary and Stock, 1994). DrRR was phosphorylated robustly, whereas AtRR responded to the treatment only weakly. Phospho-DrRR was then incubated together with ATP and either DrBphP or AgP1. In the reactions, net phosphatase activity would decrease the amount of phospho-DrRR, whereas net kinase activity would increase it. As expected, addition of AgP1 or Chimera led to an increase in phospho-DrRR when incubated in darkness, indicating kinase activity of these proteins (Figure 3C). By contrast, the addition of DrBphP decreased the amount of phospho-DrRR, especially upon red-light exposure. These findings reveal that DrBphP acts as a phosphatase with higher activity in the Pfr state than in the Pr state. The H533D mutation did not alter the phosphatase activity (Figure 3D).

The residue in the H+4 position is important for phosphatase activity of HisKA family proteins (Willett and Kirby, 2012). Indeed, the corresponding residue E356 in DrBphP appeared to have a role in the reaction as its mutation to alanine reduced the phosphatase activity (Figure 3D). Notably, the E356A variant maintained somewhat higher phospho-DrRR amounts not only in the Pfr but also in the Pr state, potentially by shielding the phospho-DrRR from spontaneous hydrolysis during the reaction. Like the H533D and wild-type variants, E356A did not show any histidine kinase activity (Figure 3E). Interestingly, the phosphatase activity in DrBphP depended on ATP addition, as we observed hardly any changes in phospho-DrRR when ATP was excluded from the reaction (Supporting Figure S3C). This is consistent with the notion that ADP, ATP, or adenosine 5'-[β,γ -imido]triphosphate (AMP-PNP) serves as a cofactor for the phosphatase activity (Zhu et al., 2000).

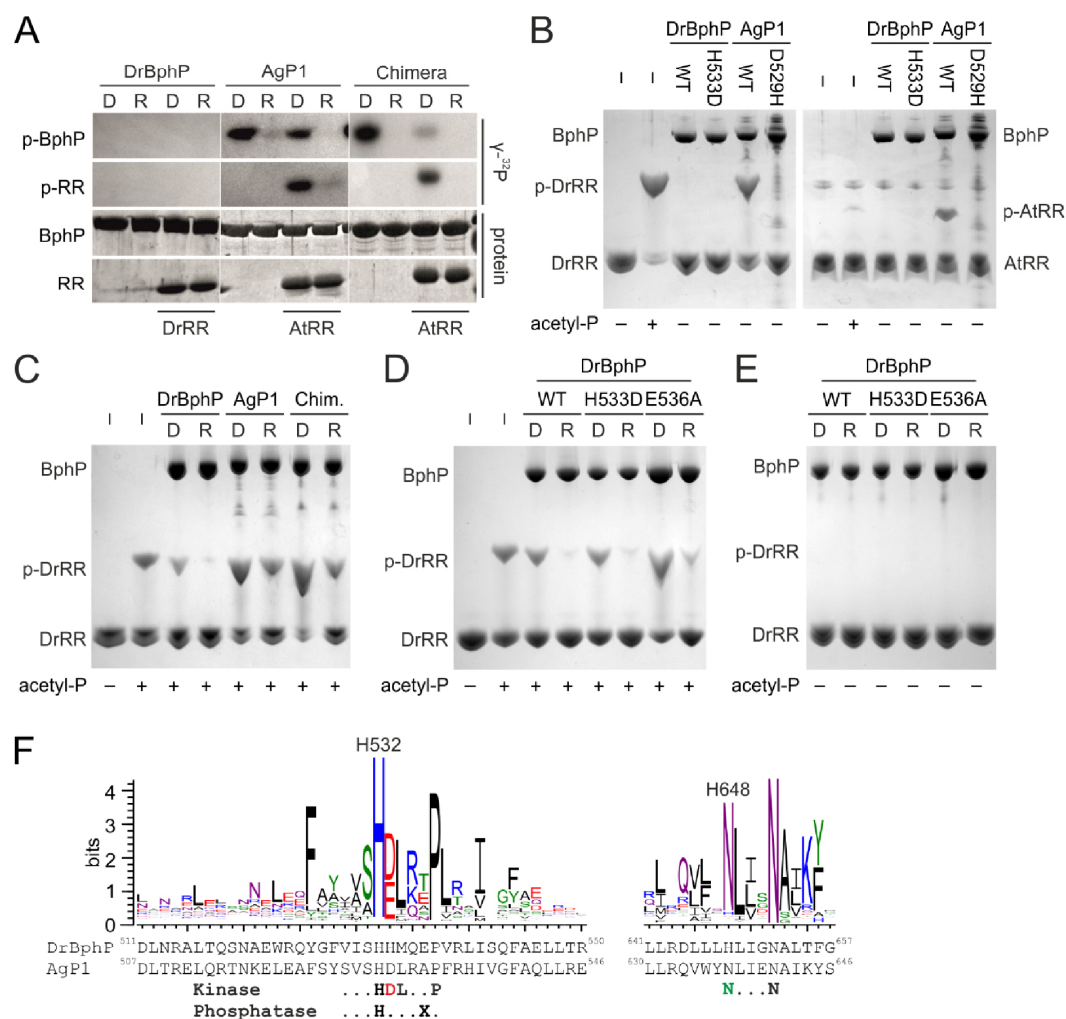


FIGURE 3. Kinase and phosphatase activity of the phytochromes DrBphP and AgP1. **A.** Kinase and phosphotransfer activity of the phytochromes, detected for radioactive phosphate (γ -³²P) and total protein. Each phytochrome sample (BphP) was incubated with γ -³²P-ATP, either with or without the response regulator (RR) protein. DrBphP was incubated with or without DrRR, whereas AgP1 and Chimera were incubated with or without AtRR. Full gel is shown in Supporting Figure S3A. **B.** Kinase activity of DrBphP, AgP1, and their variants with the H+1 residue mutated. Each well was loaded with equal amount of response regulator. The phosphorylated response regulators (denoted p-DrRR and p-AtRR) migrate slower in the gels and are therefore resolved from their unphosphorylated counterparts. **C.** Phos-tag detection of the phosphatase activity of DrBphP, AgP1 and Chimera. Equal amount of phospho-DrRR were applied to each reaction. **D.** Phosphatase activity of DrBphP and its mutants H533D and E536A. Equal amount of phospho-DrRR were applied to each reaction. **E.** All DrBphP variants were inactive kinases. Full gels are shown in Supporting Figure S3. **F.** Sequence logo of 50,000 histidine kinase sequences, shown here for the H box close to the phosphoaccepting histidine (H532 in DrBphP) and the N box in the CA subdomain. The height of each residue name indicates the amount of conservation. The protein sequence of DrBphP and the fingerprint sequence motifs are shown below the graph. In the case of phosphatase activity, the residue at H+4 position varies and is denoted as X.

DrRR crystal structure reveals a canonical response regulator dimer

As DrBphP and AgP1 strikingly differ in their enzymatic activity and interactions, we next asked whether these differences can be explained at the structural level. In particular, we speculate that these differences are down to the structure of the interface between the DHp and RR moieties. To model this interface with confidence, we solved the crystal structure of the response regulator from *D. radiodurans* (DrRR) at 2.1 Å resolution (see Table 1 for crystallographic statistics). The protein crystallized in the tetragonal P4₁2₁2 space group with four monomers in the asymmetric unit. These monomers form two ‘inverted 4-5-5 dimers’ with a dimerization interface built by a $\alpha 4$ – $\beta 5$ – $\alpha 5$ face of each monomer (Gao and Stock, 2010) (Figure 4A), similar to most other phytochrome RR structures with a receiver domain (Benda et al., 2004; Im et al., 2002; Yang et al., 2015). However, the AtRR assumes a ‘hand-in-hand’ dimer (Baker et al., 2016), in which the C-terminal extension forms an antiparallel β -strand interface with a sister monomer (Figure 4A). Overall, the structure of the DrRR is highly similar to other reported response regulators. It contains all the structural features and the conserved residues critical for its function as a receiver domain in two-component signaling (Figure 4C). These structural details along with functional results (Figure 3) verify that DrRR can function as a canonical response regulator in a two-component signaling system.

Notably, the crystal structure of DrRR contained Ca²⁺ instead of Mg²⁺ ions found in other response regulator structures (Baker et al., 2016; Benda et al., 2004; Im et al., 2002; Yang et al., 2015). The Ca²⁺ ions played a central role in this crystal form, as their replacement with Mg²⁺ did not allow crystal formation. Ca²⁺ ions occupy the active site of the DrRR in a similar way to Mg²⁺ in the AtRR structure (Baker et al., 2016). Although Ca²⁺ is chemically similar to Mg²⁺, its larger size leads to diffuse coordination of the ion in the active sites (Figure 4B) and 45% higher B-factors than when Mg²⁺ ions are modelled at the same sites. Consequently, the Ca²⁺ interactions differ between the four monomers in the asymmetric unit, being most similar to AtRR in monomer A (Baker et al., 2016). In each case, the Ca²⁺ ions are hexagonally coordinated to surrounding atoms, which involve water molecules, the side chains of Glu15, Asp16, Asn17 and the phospho-accepting Asp66, and the main-chain oxygen of Asn68 (Figure 4B).

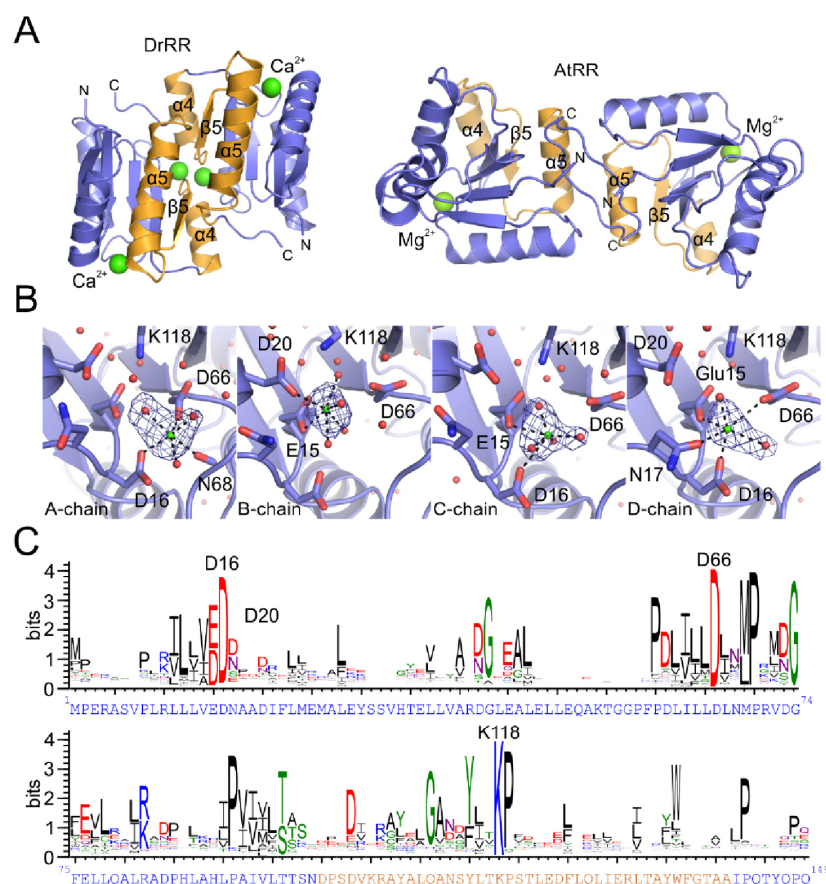


FIGURE 4. Crystal structure of the *Deinococcus radiodurans* response regulator. **A.** Cartoon representation of the dimeric DrRR and AtRR structures (PDB code 5BRJ for AtRR (Baker et al., 2016)). The $\alpha 4$ – $\beta 5$ – $\alpha 5$ face of each response regulator monomer is in orange, and the rest of the protein is in blue. Ca^{2+} and Mg^{2+} ions at the active sites, N- and C-termini, as well as dimerization helices are marked. In the case of DrRR, a dimer formed by chains A and B is shown. **B.** The active site of DrRR with its Ca^{2+} ions and interacting residues. The localization and its interactions (black dashed lines) of Ca^{2+} ions (green) differ between the chains. The omit difference ($F_o - F_c$) map of the Ca^{2+} ions are shown as blue mesh at 5.0σ . The omit maps are calculated for each monomer by repeating the final refinement step without the Ca^{2+} ion and the coordinating water molecules. **C.** Sequence logo attained from 50,000 response regulator sequences. The height of each letter indicates the amount of conservation for the corresponding amino acid (one-letter code). The key DrRR residues are shown above the graph, and the full amino acid sequence of DrRR is below the graph. The amino acid sequence is colored as in panel A.

Complex structures show different interactions in DrBphP and AgP1

To analyze how the interplay of the HK and RR proteins impacts on two-component signaling, we prepared complex models for the DrBphP/DrRR and AgP1/AtRR pairs, based on a homologous complex structure (Casino et al., 2009) and the crystal structures of the DrRR and AtRR (Baker et al., 2016). Support for the physiological relevance of the resultant structural interface also derives from a covariance analysis of cognate HK/RR pairs, as pioneered by Laub and coworkers (Skerker et al., 2008). In contrast to prior analyses, we focused on a set of hybrid receptors which comprise HK

and RR moieties in a single polypeptide chain, thus allowing to assign interacting, cognate HK/RR pairs with high confidence. The multiple alignment of several thousand such receptors revealed strong residue covariation not only within the HK and RR parts but also in between them (Gleichmann et al., 2013; Jones et al., 2012). When mapped on the structural model of the complex, strong pairwise residue covariation localized to the HK/RR interface (Supporting Figure S6), strongly speaking for realistic complex models.

To address the stability of the complex models in solution and to reveal the underlying molecular interactions, we conducted classical MD simulations at 300 K and 1 atm pressure with 0.1 M NaCl concentration using the Gromacs molecular dynamics package (Abraham et al., 2015). Over a 200 ns trajectory, both the DrBphP/DrRR and AgP1/AtRR complexes were stable. The RMSD equilibration times for protein backbone atoms were around ~60 ns for DrBphP/DrRR complex and ~80 ns for AgP1/AtRR (Supporting Figure S4), suggesting that the interactions are more defined and stronger in the DrBphP/DrRR complex. Starting from the 100 ns time point of the trajectory, we extracted snapshots at 10 ns intervals and performed extended analysis of their interactions. Representative snapshots are shown in Figure 5, all snapshots in (Supporting Figure S4).

Overall, the interactions between AgP1 and AtRR were transient and more variable than the ones in the *D. radiodurans* pair, as gauged by the larger overall RMSD values of the AgP1/AtRR complex and higher mobility of the atoms throughout the MD trajectory (Supporting Figure S4). Analysis of the snapshots in the PISA server (Krissinel and Henrick, 2007) revealed that both complex interfaces have hydrophobic core regions. The average solvation free energy upon formation of the interface indicated that this interface is more extensive in the DrBphP/DrRR complex (-11.3 kcal/M) than in the AgP1/AtRR complex (-5.8 kcal/M).

The RRs interacted mainly through their $\alpha 1$ helix (aa. 18–32 in DrRR) that aligns with the helical bundle of the four DHp helices. In addition to this main interface, DrRR has additional interactions through a loop region (aa. 119–121) that connects strand $\beta 5$ and helix $\alpha 5$. Notably, the position of this ' $\beta 5$ – $\alpha 5$ loop' and the length of the $\alpha 5$ helix differ between DrRR and AtRR, allowing DrRR more extended interactions with its phytochrome partner. The complexes contain polar interactions and well-defined salt bridges that are more pronounced in the *D. radiodurans* complex (Figure 5). Notably, Mg^{2+} ion coordinates a set of interactions between DrBphP and DrRR (Figure 5C), which are absent in the AgP1/AtRR complex (Figure 5F). We note that in DrBphP/DrRR complex, inter-chain salt bridges are less fluctuating in comparison to the AgP1/AtRR complex (Supporting Figure S5B, D).

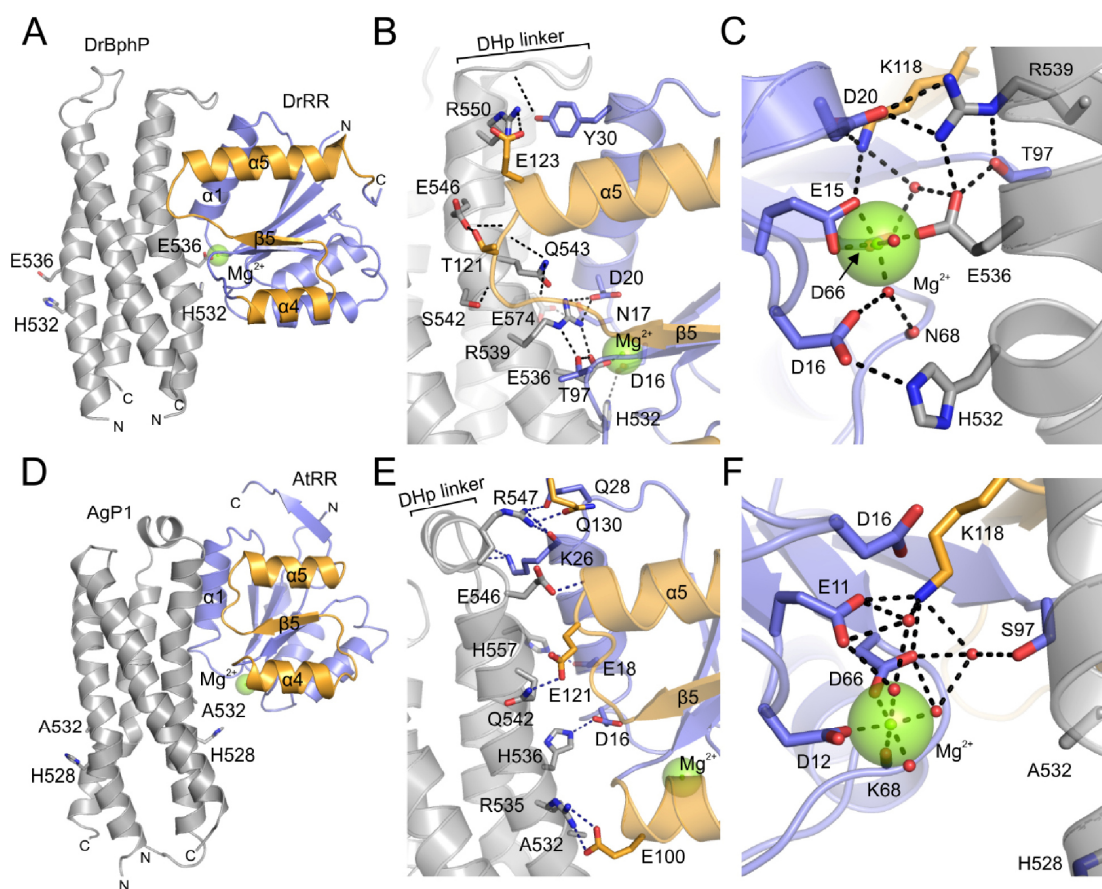


FIGURE 5. Complex models of response regulators and the interacting DHP domains. **A–C.** Structure and interactions of the DrBphP/DrRR complex, with the overall structure of the complex shown in panel A, detailed interactions in panel B, and the interaction network around the Mg^{2+} in panel C. **D–F.** Structure and interactions of the AgP1/AtRR complex, with the overall structure of the complex shown in panel D, detailed interactions in panel E, and the interaction network around the Mg^{2+} in panel F. Representative structural snapshots from the relaxation simulations are shown. Surrounding water box with Na^+ and Cl^- ions are omitted for clarity reasons, and only one monomer of the response regulator dimer is shown.

In DrBphP, the catalytic residue His532 forms a salt bridge to the DrRR residue Asp16, which orients it close to the DrRR phospho-accepting Asp66. Glu536 coordinates with Mg^{2+} and forms additional interactions with Arg539 and DrRR residue Thr97 (Supporting Figure S5A, B). The corresponding residue in AgP1 is alanine (Ala532), and therefore these interactions are absent in the *A. fabrum* pair. DrBphP residue Arg539 forms a salt bridge with DrRR residue Asp20 and interacts with Thr97, Arg539 and Glu536. The $\alpha 5$ helix is longer in DrRR than in AtRR, which enables additional contacts between the ‘ $\beta 5$ – $\alpha 5$ loop’ region (aa. 119–123) and DrBphP residues Ser542, Asn543, Glu546, and Arg550. This positioning of the ‘ $\beta 5$ – $\alpha 5$ loop’ guides the side chain of Arg539 to close proximity to Asp20, thus enabling extensive interactions around these residues (Figure 5C). In the case of AgP1, the corresponding residue Arg535 points away from Asp16 of AtRR (Figure 5E).

Additional interactions that stabilize the DrBphP/DrRR complex are mediated by DrBphP residues Glu574 and Arg550. Tyr30 of DrRR interacts with the main chain of Ala554, thus stabilizing this DHp linker loop between the two DHp helices (Figure 5B). In a similar way, AtRR residue Lys26 interacts with Ala550, Lys554 and Ser555 of AgP1, stabilizing a helical linker between the DHp helices (Figure 5E). The AgP1/AtRR complex is further supported by interactions mediated by the side chains of Gln542, Glu546, Arg547, and H557 (Figure 5E, Supporting Figure S5D).

Taken together, there are three central DrBphP residues that interact with the DrRR active site: His532, Glu536 and Arg539 (Figure 5C). These residues form a defined interaction network that includes a hexagonally coordinated Mg^{2+} ion (Figure 5C, Supporting Figure S5). The corresponding residues are missing at the active site in the AgP1/AtRR complex (Figure 5E), which leads to active-site interactions that resemble those of free AtRR (Baker et al., 2016).

DISCUSSION

The bacteriophytochrome from *D. radiodurans* is a light-activated phosphatase

Bacterial phytochromes commonly act as light-regulated histidine kinases in two-component systems (Esteban et al., 2005). Here, we introduce novel biochemical and structural insight into the activity of these phytochromes and their interaction with response regulators.

AgP1 acts as a red light-repressed histidine kinase that phosphorylates its cognate response regulator AtRR (Lamparter et al., 2002) and that from *D. radiodurans* (Figure 3A, B). Similar cross-reactivity has been reported for a bacteriophytochrome from *Pseudomonas syringae* (PsBphP) which can also phosphorylate DrRR (Bhoo et al., 2001). Therefore, it is possible that AgP1 acts promiscuously and phosphorylates other response regulators in bacteria. By contrast, DrBphP did not show any kinase activity but functions exclusively as a phosphatase (Figure 3). Therefore, DrRR is largely phosphorylated non-enzymatically or by other histidine kinases in bacterial cell under dark conditions. DrBphP can dephosphorylate phospho-DrRR upon red-light exposure and thereby elicit physiological responses. Notably, the precise function of DrBphP has been under debate ever since its role in the control of carotene production was reported (Davis et al., 1999). Our results now settle this long-standing debate and show that DrBphP is a biochemically active protein that dephosphorylates the DrRR, rather than phosphorylating it.

We fused the DrBphP PSM with the histidine kinase effector of AgP1 (Figure 1A) to produce a functional histidine kinase chimera (Figure 3C). We thus show that the DrBphP PSM is principally capable of controlling both histidine kinase and phosphatase activities in dependence of light. Consistent with this observation, the well-studied conformational changes of the DrBphP PSM during the Pr-to-Pfr transition (Burgie et al., 2014; Burgie et al., 2016; Takala et al., 2014a) are generally very similar across various phytochromes (Bjorling et al., 2015), indicating that both histidine kinase and phosphatase modules can be controlled with other phytochrome photosensory modules.

Even prior to the present elucidation of the enzymatic activity of DrBphP, its PSM has provided a versatile building block for novel light-controllable enzymes to be used in optogenetics. Pertinent enzymes have for instance been constructed through fusion of the DrBphP PSM with a cyclic-mononucleotide phosphodiesterase (Gasser et al., 2014; Stabel et al., 2019), a guanylate/adenylate cyclase (Ettl et al., 2018; Stüven et al., 2019), and a tyrosine kinase (Leopold et al., 2019). Our study introduces a red light-regulated HK chimera and phosphatase as a potential addition to the optogenetic toolkit.

The binding modes of AgP1 and DrBphP support different functionalities

Phytochrome photoactivation entails large-scale structural changes in the photosensory module, which are then relayed to the output module (Burgie et al., 2014; Burgie et al., 2016; Takala et al., 2014a). Although the molecular details are still under debate and may differ between receptors, the conformational changes in the DHP bundle likely include rotation, bending, or changes in register of the constituent helices (Möglich, 2019). These conformational transitions can then change the interactions and/or enzymatic activity of the output HK domain.

The binding of DrBphP and AgP1 to their cognate response regulators differ from each other, which may be integral to their respective activity profile. This difference manifested in dark reversion (Figure 1B), in SEC profiles (Supporting Figure S1), and in SPR and ITC analyses (Figure 2). The binding of AtRR to AgP1 was weak and transient, but the binding of DrBphP to DrRR had moderate affinity ($K_D \sim 10 \mu\text{M}$) and slower association kinetics. Our structural data indicate that the binding interfaces in both complexes are generally similar but differ in their details: The DrBphP/DrRR complex had relatively stable interactions, whereas the interactions appeared transient and less defined in the AgP1/AtRR complex (Figure 5).

We did not detect clear light-induced change in binding in the BphP/RR pairs, which is consistent with structural evidence that the RR binds to the DHP domain in a similar way regardless of whether the receptor resides in the kinase or phosphatase state. Notably, the relevant interaction epitope of the DHP domain experiences only minor structural changes upon HK (in)activation (Möglich, 2019; Trajtenberg et al., 2016). The CA domain on the other hand binds to a DHP region that varies upon light activation. This may lead to a change in kinase activity, either through a difference in CA binding, through varying the accessibility of the catalytic histidine (Trajtenberg et al., 2016), or both. The binding sites of the CA domain and RR partially overlap, as also visible in the covariance analyses of the interfaces (Supporting Figure S8), which should create competition between the two binding schemes.

The structural changes in the DHP domain may facilitate the switch between the CA binding during the autokinase reaction and the RR binding during the phosphotransfer or phosphatase reaction. As the RR competes with the CA domain for binding to the DHP domain, transient interactions between the molecules would be favored in the kinase reactions. The alternating binding of CA and RR may also be facilitated by structural asymmetry observed for several HKs in their kinase-active state (Bhate et al., 2015; Jacob-Dubuisson et al., 2018; Möglich, 2019). Although the phosphatase reaction is greatly facilitated by a CA domain (Zhu et al., 2000) and ATP (Supporting Figure S3C), the CA

binding during the phosphatase reaction likely differs from that of kinase reaction. This difference may allow relatively slow binding kinetics between the DHp bundle and the phosphate-presenting response regulator.

Two residues in the DHp helix govern the HK activity

Two residues within the DHp domain, at positions +1 and +4 relative to the active-site histidine, have been implicated as particularly important for the enzymatic activity in the HisKA family. First, the autophosphorylation reaction involves a nucleophilic attack by the histidine to the γ -phosphate of ATP. This is facilitated by an acidic residue (aspartate or glutamate) in the H+1 position acting as a general base (Casino et al., 2014). Second, a threonine or asparagine residue in the H+4 position governs phosphatase activity (Willett and Kirby, 2012). There is no crosstalk between the residues, as the H+1 position does not contribute to the phosphatase activity and H+4 position is not required for the kinase activity (Willett and Kirby, 2012).

A large-scale sequence analysis of histidine kinases shows that the acidic residue in the H+1 position is strictly conserved among the HisKA family (Figure 3F), underlining its importance. If this residue is mutated, the kinase activity is impaired, as indicated by our results on the D529H mutant of AgP1 and wild-type DrBphP (Figure 3B). A histidine in the H+1 position is very rare among the HK sequences (Supporting Figure S7). Although important, the activity of DrBphP could not be rescued only by introducing an aspartate to this H+1 position (Figure 3B, E). In HisKA proteins, the acidic residue in H+1 is accompanied by an asparagine in the N-box of the CA domain, which stabilizes the active HK conformation and participates in phosphoryl transfer from ATP to the catalytic histidine (Herrou et al., 2017). Indeed, this asparagine shows a high level of conservation within the HisKA family (Figure 3F). Our model of AgP1 HK supports this interaction between H+1 aspartate (Asp529) and the N-box asparagine (Asn637) (Figure 6A). By contrast, this interaction is likely absent in DrBphP, as both these residues are histidines (His533 and His648) (Figure 6A). Consistent with this observation, the sequence analysis indicates that when the H+1 position is a histidine, the conservation of the N-box asparagine is lost (Supporting Figure S7A).

DrBphP has Glu536 in the H+4 position, indicating that this residue is central for the phosphatase activity. As previous studies on the phosphatase activity in HisKA proteins have mainly concentrated on threonine and asparagine residues, not much is known about the role of glutamate at this position (Brandon et al., 2000; Dutta et al., 2000; Ji et al., 2020; Schramke et al., 2016; Willett and Kirby, 2012). That notwithstanding, glutamate is as conserved at this site as threonine or asparagine (Figure 3F), which implies that all these residues play equally important roles, potentially fine-tuning the extent of the phosphatase reaction. Changing this residue to alanine diminishes or removes the phosphatase activity (Dutta et al., 2000; Ji et al., 2020; Schramke et al., 2016; Willett and Kirby, 2012), like demonstrated presently in the E536A variant of DrBphP and the wild-type AgP1 (Figure 3D).

In our DrBphP/DrRR complex model, Glu536 forms a distinctive interaction with Mg^{2+} in the active site (Figure 5C). As this site would be occupied by the phosphate moiety of the phospho-DrRR, it is likely that Glu536 facilitates the dephosphorylation reaction. Accordingly, the side-chain hydroxyl

group of the threonine or asparagine at the H+4 residue may retain similar interactions as the carboxyl group of the glutamate. In addition to Glu536, Arg539 in the H+7 position participates in the interactions at the DrRR active site (Figure 5C), suggesting a subsidiary role in the phosphatase reaction. This is supported by similar conservation patterns of the H+7 arginine and the H+4 glutamate in HisKA proteins (Supporting Figure S7A).

Model for bacteriophytochrome photoactivation

To conclude, Asp529 in the H+1 position enables AgP1 to function as a histidine kinase in the Pr state. In the case of DrBphP, His533 at H+1 (along with other residues) renders it inactive as a Pr-state kinase, whereas Glu536 at the H+4 position makes it an effective phosphatase in the Pfr state (Figure 3). Thus, the Pr-like conformation of the bacteriophytochromes in general supplies a structural framework for kinase activity, whereas Pfr-like conformation shifts the output activity to a phosphatase (Figure 6B). Indeed, many HisKA family proteins function as both kinases and phosphatases, and these modes of action can likely be switched by a change of the relative orientation of DHp helices (Trajtenberg et al., 2016). As a case in point, in the related histidine kinase YF1, blue light prompts quaternary transitions that channel into a register shift and supercoiling of the DHp helices (Berntsson et al., 2017; Ohlendorf et al., 2016). Both kinase and phosphatase activities are therefore supported by changes within the same structural framework. The bidirectional activity would also require both sets of activity-determining residues in the HK domain, which seems to be the case in many phytochromes (Supporting Figure S7B).

Phytochrome function includes several structural tiers that range from the chromophore surroundings to large-scale structural changes in the entire protein. These tiers are in dynamic equilibrium, which can be shifted by the other tiers or by external factors (Takala et al., 2018). The level of phytochrome output activity can be considered to be in an equilibrium between histidine kinase and phosphatase activities (Bhate et al., 2015; Möglich, 2019) (Figure 6B). This equilibrium can be shifted to one direction by the light-induced changes in the photosensory module, and tuned by the sequence variation in the HK domain. In this study, we have shown how small differences in sequence dictate opposing enzymatic activities in two canonical phytochromes. In both cases, light controls their enzymatic activity.

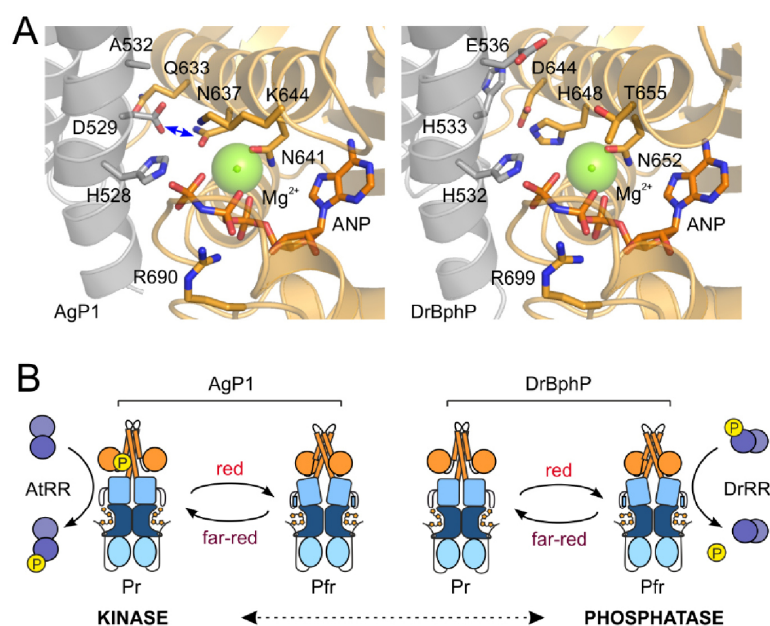


FIGURE 6. Structural comparison of residues important for the phosphotransfer reaction. **A.** Homology models of AgP1 and DrBphP that are based on the histidine kinase domain structure of HK853/EnvZ chimera in its phosphotransfer state (PDB 4KP4) (Casino et al., 2014). The central residues are denoted as sticks, dimerization and phosphohistidine (DHp) domain is shown in grey, and the catalytic ATP-binding (CA) domain is shown in orange. In AgP1, the potential interaction between His528 and Asn637 is shown as blue double-headed arrow. **B.** Proposed kinase and phosphatase activities of AgP1 and DrBphP. Many histidine kinases can function as both kinases and phosphatases, which may be governed by their DHp orientations (Trajtenberg et al., 2016). This transition between the two states is illustrated here as vertical line. In the case of the two phytochromes studied here, red light illumination causes conformation that favors phosphatase activity. In resting (Pr) state, the HK conformation favors kinase activity.

EXPERIMENTAL PROCEDURES

Cloning and DNA material. The phytochrome from *Deinococcus radiodurans* strain R1 (DrBphP, gene DR_A0050) in pET21b(+) plasmid (Novagen) was kind gift from Prof. Richard Vierstra (Davis et al., 1999; Wagner et al., 2007), and phytochrome 1 from *Agrobacterium fabrum* strain C58 (AgP1, gene Atu1990) in pQE12 (Qiagen) was a kind gift from Prof. Tilman Lamparter (Lamparter et al., 2002). AgP1 has a spontaneous R603C mutation, which resides on the surface of the CA domain. The mutations to DrBphP (H533D, E536A) and for AgP1 (D529H) were introduced with QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). For cloning the chimera construct, DrBphP residues 513–755 were replaced with Agp1 residues 509–745. First, an XhoI restriction site was introduced after DrBphP residue 513 with QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). Then, the C-terminal Agp1 fragment (aa 511–755) was ligated between the new XhoI site and an XhoI site right before the C-terminal His₆-tag. After introduction of the Agp1 fragment, the new XhoI site was changed to Agp1 residues 509–510 by site-directed mutagenesis. The response regulators from *Deinococcus radiodurans* strain R1 (DrRR, gene DR_A0049) and *Agrobacterium fabrum* strain C58 (AtRR, gene Atu1989) (Bhoo et al., 2001) were produced as a service (Invitrogen). The response regulator constructs were cloned into pET21b(+) vectors (Novagen) by using restriction sites BamHI and XhoI. The EGFP-RR constructs were prepared with Gibson assembly cloning, in which N-terminal T7 tag was replaced with an EGFP-C1 sequence (Polstein and Gersbach, 2012). In addition, a linker of 10 residues (DSAGSAGSAG) was introduced with primers between the RR and EGFP sequences.

Sample expression and purification. All DrBphP variants and the response regulators were expressed in *Escherichia coli* strain BL21 (DE3) overnight at 20–24 °C, and purified with HisTrap affinity purification followed by size-exclusion chromatography like previously described (Takala et al., 2016). The His₆-tagged phytochrome samples were first purified with NiNTA affinity purification using HisTrap™ columns (GE Healthcare), followed by size-exclusion chromatography (HiLoad™ 26/600 Superdex™ 200 pg, GE Healthcare) in buffer (30 mM Tris, pH 8.0). No external biliverdin was added to the cell lysate in response regulator purifications. AgP1 and its D529H mutant were expressed in NEB Express® I^a *E. coli* strain (New England Biolabs). The purification protocol was identical to other samples with a couple of exceptions: Protease inhibitor mix (ROCHE) and 0.5 mM TCEP were included in the sample before lysis, and affinity purification was conducted in (30 mM Tris/HCl, 150 mM NaCl, 1 mM TCEP) and varying imidazole concentration (5–500 mM). All purified protein samples were concentrated to 25–30 mg/ml in (30 mM Tris/HCl, pH 8.0) and flash-frozen.

Absorption spectroscopy. The dark reversion of the phytochromes was measured by the absorption spectroscopy using Agilent Cary 8454 UV-Visible spectrophotometer (Agilent). Absorption spectra in the wavelength range of 690–850 nm were recorded from the mixture of response regulator and Pfr-populated BphP sample. The BphP samples were first diluted to 1.0 μM in (25 mM Tris/HCl, pH 7.8, 5 mM MgCl₂, 4 mM 2-mercaptoethanol, 5% ethylene glycol) to obtain an approximate A₇₀₀ value of 0.1 cm⁻¹. Ten times concentration (100 μM) of cognate response regulator was added into the BphP sample. Then the phytochromes were driven to a maximum population of the Pfr state by saturating illumination with 665 nm LED for 3 min, followed by immediate data acquisition in dark.

Dark reversion data were recorded at 1 min intervals for first 10 min, which was followed by intervals of 5 min up to 1 hour and finally 10 min intervals until 2 hours. All measurements were performed in dark at ambient conditions (room temperature). The steady-state spectra of Pr- and Pfr-state samples, in presence or in absence of cognate response regulator, were measured in the same buffer as for dark reversion. Pr state spectra were measured from the dark-adapted samples while Pfr spectra were measured after 3 min illumination with 665 nm LED.

The exponential fits from dark reversion data were calculated with Matlab (MathWorks inc.) using Equation 1. In the case of DrBphP samples, three components were used for fitting, whereas two components were adequate for the rest of the samples.

$$\frac{A_{750}}{A_{700}}(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3} \quad (1)$$

Where t is time, A_{700} and A_{750} are absorption values at specified wavelength, A_n is the decay amplitude of the absorbance-ratio, and τ_n the time constant of the decay component.

Size-exclusion chromatography (SEC) with multiwavelength detection. The size-exclusion measurements (Supporting Figure S1) were conducted as previously described (Takala et al., 2014b). For sample separation, a Nanofilm SEC-250 (300 mm × 4.5 mm) column (Sepax Technologies, Delaware, US) was used with (25 mM Tris/HCl pH 7.8, 5 mM MgCl₂, 4 mM 2-mercaptoethanol, 5% ethylene glycol) as a mobile phase. Experiments were executed with 350 µL/min flow-rate using the Shimadzu HPLC VP10 pumping system (Shimadzu Corporation, Kyoto, Japan). The eluant was detected at 1.5625 Hz with a diode array UV-Vis detector (SPD-M10A, Shimadzu) and at 2.00 Hz with a fluorescence detector (RF-10A_{XL}, Shimadzu). For each run, 20 µL of sample mixture was injected briefly after pre-illumination with 785 nm or 655 nm light. The protein concentrations used were 6 µM (EGFP-DrRR), 30–50 µM (DrBphP), AgP1 60 µM (AgP1), and 1 µM (EGFP-AtRR). To exclude spectral overlap between the samples, 700 nm absorption was used for BphP detection and 488/509 nm excitation/emission was used for EGFP-RR detection. The Gel Filtration standard (Bio-Rad) was used according to the manufacturer's instructions. The molecular weight estimates were determined by calculating a standard curve of marker proteins Vitamin B12 (1.35 kDa) myoglobin (17 kDa), ovalbumin (44 kDa), γ-globulin (158 kDa), and thyroglobulin (670 kDa).

Surface plasmon resonance (SPR). For surface plasmon resonance measurements, phytochrome samples were dialyzed overnight to (20 mM HEPES, 300 mM NaCl₂, 5 mM MgCl₂, 0.10% (v/v) Tween20, pH 7.5) with a Spectra/Por® Micro Float-A-Lyzer Dialysis Device (Spectrum Laboratories, USA). The measurements performed using Biacore X instrument (GE Healthcare). Response regulators were coupled onto carboxymethyl-dextran hydrogel-coated SPR Sensorchip (XanTec bioanalytics GmbH) according to manufacturer instructions. Each response regulator was coupled onto chip surface as 3 mg/mL (150 µM) in an acetate buffer (20 mM sodium acetate, pH 4.2) using EDC/NHS coupling protocol. The remaining activated groups on the sensor chip were then quenched with (1 M ethanol-amine-HCl, pH 8.5). The measurements were conducted by injecting 40 µL phytochrome sample at 20 µL/min, followed by wash step with (20 mM HEPES, 300 mM NaCl₂, 5 mM MgCl₂, 0.10% (v/v) Tween20, pH 7.5). Samples were either pre-illuminated with far-red (785

nm) or red (655 nm) LED light before injection, and all measurements were done under dim light at room temperature.

The sensorgrams were analyzed using the BIAevaluation-software version 4.1 (Biacore Life Sciences). The sharp peaks corresponding to the injection start (0 s) and stop (120 s) in each sensorgram were excluded from the analysis. For kinetic fit, a simple 1:1 interaction model between analyte and immobilized ligand was applied, followed by simultaneous fit of k_a/k_d kinetics. The model is equivalent to the Langmuir isotherm for absorption to a surface. Steady state binding levels (R_{eq}) were obtained by fitting a horizontal straight line to a chosen section of the sensorgrams (blue lines in Figure 5) and determining the average response. R_{eq} values (y) and concentrations (x) were plotted in Origin 2018b and a nonlinear simple fit was obtained using the following Equation 2 where A stands for concentration at R_{eq} .

$$R_{eq} = (A)R_{max} = (A) + K_D \quad (2)$$

Isothermal calorimetry (ITC).

Isothermal calorimetry was conducted with MicroCal PEAQ-ITC (Mavern Pananalytical, United Kindom). For the measurements, the purified protein (in 30 mM Tris/HCl pH 8.0) were diluted 1:1 with 2x (50 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 8 mM 2-mercaptoethanol, 10% ethylene glycol). BphP (30–50 μ M, 300 μ L) was loaded in the sample cell and RR (750–800 μ M, 75 μ L) was loaded in the injection syringe. To verify the Pr state of the BphP samples, they were briefly illuminated with 785 nm LED light just before sample application to the cell. The system was equilibrated to 25 °C with a stirring speed of 750 rpm in dark. Injection scheme started with a 0.4 μ L response regulator injection, followed by 2 μ L injections every 150 s. The ITC measurement in (30mM Tris/HCl, pH 8.0) were made using a Micro-200 ITC (MicroCal, Malvern). The concentrations used were 170–250 μ M (BphP) and 750–800 μ M (RR). BphP sample (206 μ L) was loaded into the sample cell and RR (70 μ L) was loaded into the injection syringe. The system was equilibrated to 25 °C with a stirring speed of 750 rpm. The injection scheme started with a 0.2 μ L injection followed by 2 μ L injections every 180 s. In both measurements, background signal was estimated by injection of response regulator into buffer and buffer into phytochrome with the same parameters. All data from triplicate experiments were analyzed using ORIGIN 7-based MicroCal PEAQ-ITC Analysis Software (Malvern Panalytical). The curves were fitted into a single-site binding isotherm with the first injection excluded. The K_D value was reported as $\pm$ SD from three repeats.

Radiolabeled kinase assay. The radiolabeled kinase assay was done in a similar way to Lamparter et al. (Lamparter et al., 2001; Lamparter et al., 2002). Purified BphPs and RRs were diluted to approximate concentrations of 3.5 μ M (0.3 mg/ml) and 9 μ M (1.7 mg/ml), respectively, in (25 mM Tris/HCl pH 7.8, 5 mM MgCl₂, 4 mM 2-mercaptoethanol, 5% ethylene glycol), and pre-illuminated briefly with saturating 785 nm LED light. Reaction was started by adding 3.7 kBq of [γ -³²P]ATP (PerkinElmer) in a total reaction volume of 10 μ L. The samples were then incubated at 25 °C either in dark or under constant 655 nm LED illumination (5 mW/cm²) for 20 min. The reaction was stopped by adding SDS sample buffer. The samples were then separated on 12% SDS-PAGE, and the gels were stained with Serva Blue, followed by drying in vacuum drier. The dry gels were then

photographed and their radioactivity was monitored with an X-ray film. The experiment was repeated three times.

Protein phosphorylation by Acetyl phosphate and Phos-Tag detection. In order to create phosphorylated response regulator we adapted the method described by McCleary and Stock (McCleary and Stock, 1994). There, response regulators (2–3 μg) were incubated with 50 mM acetyl phosphate for 30 min. The reactions were conducted at 37 °C in (25 mM Tris/HCl pH 7.8, 5 mM MgCl_2 , 4 mM 2-mercaptoethanol, 5% ethylene glycol), followed by buffer exchange to (30 mM Tris/HCl, pH 8.0) with Vivaspin centrifugal concentrator (Sartorius, Germany). The final phosphoprotein concentrations were adjusted to 1.5 mg/ml (80 μM). Both kinase and phosphatase reactions were conducted in (25 mM Tris/HCl pH 7.8, 5 mM MgCl_2 , 4 mM 2-mercaptoethanol, 5% ethylene glycol), where all the desired proteins (2–4 μg each) were incubated in 10 μl total volume at 25 °C, with or without 1mM ATP. The reactions were started by adding ATP to the mixture and incubated either in dark or under saturating 657 nm red light. After 20–30 min, the reactions were stopped by adding 5x SDS loading buffer. For the mobility shift detection of phosphorylated proteins, we applied Zn^{2+} -Phos-tag® SDS-PAGE assay (Wako Chemicals). The 9% SDS-PAGE gels containing 25- μM Phos-tag acrylamide were prepared, and 10 μl of each reaction were run at 40 mA/gel at room temperature according to manufacturer instructions.

Crystallography. DrRR was crystallized with hanging drops vapor diffusion method. The protein of 10 mg/ml concentration was mixed in a 1:1 ratio with reservoir (0.1 M HEPES pH 7.5, 0.3 M CaCl_2 , 25% PEG400). Crystals formed in few days and were flash-frozen in the reservoir solution containing 15% glycerol. The diffraction data were collected with 0.873 nm wavelength in beamline ID23-2 at the European Synchrotron Radiation Facility (ESRF). The data were processed with the XDS program package version January 26, 2018 (Kabsch, 1993). The crystals belonged to space group P41212 with two dimers in an asymmetric unit. The initial phases were solved by molecular replacement with Phaser (McCoy et al., 2007). As for a search model, a DrRR homology model was produced on-line with SWISS-MODEL workspace (Arnold et al., 2006; Biasini et al., 2014) and a crystal structure of a cyanobacterial response regulator RcpA (PDB code 1K68) as a template (Benda et al., 2004). The structure was further refined with REFMAC version 5.8.0135 (Murshudov et al., 2011) with automatic weighting and automatically generated local NCS restraints. The model building was done with Coot 0.8.2. (Emsley et al., 2010). For the final refinement cycles, six TLS regions for each protein chain were implemented from the TLS Motion Determination (version 1.4.0) web server (Painter and Merritt, 2006). The final structure had $R_{\text{work}}/R_{\text{free}}$ of 0.181/0.218. Statistics from data collection and refinement can be found in Table 1. Figures from crystal structures and complex models were created with the PyMOL Molecular Graphics System version 2.3.3 (Schrödinger, LLC).

Table 1. Crystal data collection and processing statistics.

Data Collection^a	
Space group	P 41 21 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	87.65, 87.65, 181.21
α , β , γ (°)	90.00, 90.00, 90.00
Resolution (Å)	49.74–2.0 (2.15–2.10) ^a
<i>R</i> _{merge}	0.172 (2.882)
CC _{1/2}	0.999 (0.503)
<i>I</i> / σ (<i>I</i>)	12.01 (1.09)
Completeness (%)	100.0 (100.0)
Redundancy	4.13 (3.94)
Wilson B factor	49.54
Refinement	
Resolution (Å)	49.74–2.10 (2.15–2.10) ^a
No. of reflections	39,970 (2,891) ^a
<i>R</i> _{work} / <i>R</i> _{free}	0.181/0.218 ^b (0.330/0.318)
Overall B factor	59.84
No. of atoms	
Protein	4,389
Heterogen ^c	9
Water	240
Geometry	
RMSD	
Bond lengths (Å)	0.013
Bond angles (°)	1.811
Ramachandran	
Favored (%)	96
Allowed (%)	16
Outliers (%)	5
PDB Code	6XVU

^a Outer shell values used in the refinement are in parentheses.

^b Test set for *R*_{free} calculation constitutes 5% of total reflections that were randomly chosen.

^c This includes nine Ca²⁺ atoms.

Computational modelling. For computational simulations, DrBphP/DrRR and AgP1/AtRR complexes were constructed based on a crystal structure containing a sensor histidine kinase and its response regulator from *Thermotoga maritima* (PDB: 3DGE) (Casino et al., 2009). Homology models consisting the dimeric DHp bundle of DrBphP (aa 520–592) and AgP1 (aa 513–584) were created on-line with SWISS-MODEL workspace (Arnold et al., 2006; Biasini et al., 2014) by using the corresponding DHp part of the *T. maritima* histidine kinase (aa 248–316) as a template structure (Casino et al., 2009). As for the response regulators, the crystal structures AtRR response regulator (PDB: 5BRJ) (Baker et al., 2016) and DrRR (this paper) were applied as dimers. Waters that clashed

with the interface and the phosphates at the active sites were not included in the models, whereas the Ca^{2+} and Mg^{2+} ions from the response regulator structures were left intact.

Gromacs 2018.8 (Abraham et al., 2015) classical molecular dynamics package has been used to perform further modelling and simulations. Both AgP1/AtRR and DrBphP/DrRR complexes has been converted into the Gromacs topology, solvated within 15x15x15 nm periodic cubic box of water and neutralized with counterions. In case of DrBphP/DrRR complex we have replaced Ca^{2+} ions which resides in DrRR crystal structure with Mg^{2+} to be consistent with kinetic studies in solution. Additional Na^+ Cl^- ions has been added to the neutralized cell in order to achieve 0.1M total concentration of salt. Amber03 (Duan et al., 2003) forcefield has been used for the proteins while water has been modelled with TIP3P (William et al., 1983) parameters.

Classical molecular dynamics simulations have been performed using the following protocol: at first we have minimized our systems for 10000 steps with steepest descent method. Then 200 ns of Classical MD simulation has been performed within NVP ensemble at 300K temperature using a V-rescale thermostat with 0.5 ps time constant (Bussi et al., 2007) and at 1 atm pressure using Parrinello-Rahman barostat with 1 ps time constant (Parrinello and Rahman, 1981). All bond lengths have been constrained to their equilibrium values, taken from the force field parameters with LINCS method (Hess et al., 1997), which allowed us to use 0.2fs time-step for the trajectory integration. A particle mesh Ewald (PME) method (Essmann et al., 1995) has been used to account for periodic electrostatic interactions with real-space cutoff of 1.5 nm, while Lennard-Jones non-bonded interaction has been treated with a cut-off scheme using a range of 1.5 nm. RMSD of the backbone and RMSF of all the atoms of the proteins with respect to the initial configuration have been extracted from the trajectory.

Starting at 100 ns we have extracted snapshots each 10 ns and performed an extended analysis of interaction between kinases and response regulators. The interactions within the complex structures were analyzed with 'Protein interfaces, surfaces and assemblies' (PISA) service at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel and Henrick, 2007). In addition, most prominent contacts have been analyzed by plotting contact distances throughout the MD simulation using the standard Gromacs trajectory tools.

Sequence analysis. To analyze sequence conservation and covariance in sensor histidine kinases, we conducted a BLAST search for the DHp and CA domains (residues 511–755) of DrBphP (Uniprot id BPHY_DEIRA, WP_010889310.1) against the non-redundant (nr) protein sequence database. Using the Biopython interface (Cock et al., 2009) and custom Python scripts (Yee et al., 2015), we retrieved the top 50,000 sequence hits, corresponding to an *E*-value cutoff of 8.1×10^{-15} . The sequences were clustered at a 50% identity level with UCLUST (Edgar, 2010), and the 5,404 cluster centroid sequences were determined. The original search sequence (WP_010889310.1) was added, and the sequences were aligned using MUSCLE (Edgar, 2004). The consensus sequence of the alignment, mapped onto the search sequence, was plotted with WebLogo v3.6.0 (Crooks et al., 2004). Based on the alignment, covariance analysis was conducted with PSICOV (Jones et al., 2012) as described before (Gleichmann et al., 2013). Using custom Python scripts, the score matrix was plotted, and pairwise scores above a cutoff of 0.6 were plotted onto a homology model of the DHp and CA

domains of DrBphP (Figure 6A, Supporting Figure S6D). Homology models of DrBphP and AgP1 HK were calculated using SWISS-MODEL (Waterhouse et al., 2018) based on the HK853/EnvZ chimera in its phosphotransfer state (PDB 4KP4) (Casino et al., 2014).

The sequence analysis of the response regulator proteins was carried out similarly. A BLAST search for the sequence of *D. radiodurans* RR (Q9RZA5_DEIRA, WP_010889309.1) provided 50,000 sequences with an *E*-value cutoff of 2.5×10^{-10} . Clustering at 50% identity yielded 4,338 sequences, to which were added those of the DrRR (WP_010889309.1) and AtRR proteins (Q7CY46_AGRFC, WP_121650967.1). Sequence alignment and logo representation were done as for the histidine kinase data.

For the analysis of covariance between the histidine kinase and the RR (Supporting Figure S6), the above BLAST hits were scanned for proteins containing consecutive DHp, CA and RR domains in a single polypeptide chain. To be included in the subsequent analysis, entries were considered if they contained the Pfam HSKA, HSKA_2 or HSKA_3 domains (Finn et al., 2006), immediately followed by HATPase_c and Response_reg domains, with each domain not separated by more than 50 residues at maximum. The resultant 6,805 sequences were clustered at 50% identity, which left 5,386 centroid sequences. The amino acid sequences of the DrBphP (residues 511–755) and DrRR were concatenated and added. All sequences were then aligned as above and analyzed by PSICOV (Jones et al., 2012). Pairwise scores above a cutoff of 0.6 were plotted onto a structural model of the DrBphP/DrRR complex (Supporting Figure S6C). In a control run, the aligned sequences were split into their DHp/CA and RR parts and randomly recombined before the analysis by PSICOV. The scrambling of the alignment abolished covariation between the DHp/CA and RR parts (Supporting Figure S6A–B).

ACCESSION NUMBERS

The Protein Data Bank accession number for the structure reported here is 6XVU.

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SUPPLEMENTAL DATA

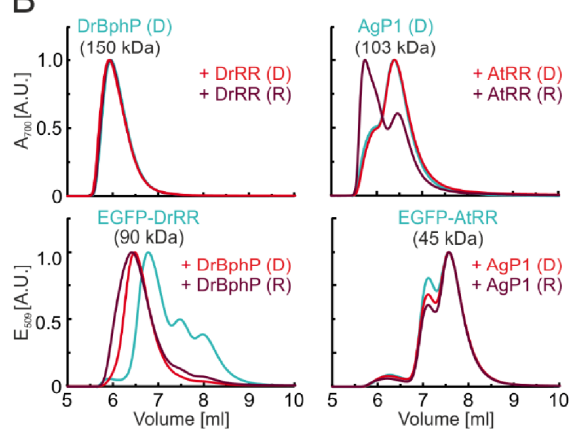
Supplemental Data include seven Supporting Figures S1–7.

Supporting Figure S1. Evaluation of dark reversion data and full gels for radiolabeled kinase assay and size-exclusion chromatography of BphP/RR pairs. **A.** The dark reversion data (Figure 1B) were approximated with an exponential fits. Table summarizes the time constants (τ_n) and decay amplitudes (A_n) of the reversion with and without response regulator. Satisfactory fits were obtained by using a sum of three decay components, resulting root mean square error (RMSE) values of 0.003–0.006. $A_{750/700}$ denotes the maximum absorption ratio at $t = 0$ min. The dark reversion of AgP1 was similar to previously published results, where three components were used (Bai et al., 2016). DrBphP however had a faster dark reversion rate and different time constants than reported before (Takala et al., 2015; Takala et al., 2014b). We therefore note that the differences in experimental parameters, including the buffer system, may cause the discrepancies in the reversion rates. Although the comparison between other studies is therefore problematic, the reversion rates within this study are fully comparable. Addition of the DrRR affected only the third time constant of the DrBphP reversion, from 480 minutes to 140 minutes and their relative amplitudes, which means that the structural properties of the DrBphP that are responsible for the third reversion component is affected by the DrRR binding. **B.** Size-exclusion chromatography of DrBphP and AgP1 and their response regulators as EGFP fusions. In order to resolve the phytochromes from response regulators, we fused the response regulators with EGFP. These EGFP-RR proteins could be exclusively detected with 509 nm emission, whereas phytochrome retention could be detected with biliverdin-specific 700 nm absorption. Interacting proteins from *D. radiodurans* (DrBphP and EGFP-DrRR) are in left, and the proteins from *Agrobacterium fabrum* (AgP1, EGFP-AtRR) are in right. BphP retentions are plotted at 700 nm absorption (upper panels) and EGFP-RR retentions are plotted as 509 nm fluorescence (lower panels). **C–D.** Size-exclusion chromatography of phytochromes and their response regulators in two different buffers: 30 mM Tris/HCl, pH 8.0 (C), and 67 mM phosphate, 200 mM NaCl, pH 8.0 (D). The experiments were conducted at 4°C with Superdex 200 Increase 3.2/300 (GE Healthcare). Protein retentions were detected at 280 nm absorption. The void volume (1.00 ml) was verified with Dextran, and the molecular weights in C were estimated from standard curve attained from Gel Filtration Calibration Kit LMW (GE Healthcare). Each protein was injected at 10 mg/ml concentration in 30 μ l. The retention graphs show that DrRR remained monomeric in both conditions, whereas AtRR was mainly a dimer. DrBphP behaved like previously described (Takala et al., 2014b): Red light illumination led to a second phytochrome peak that elutes right after the main Pr peak (C). However, DrBphP readily oligomerized once NaCl is included, especially after red light illumination (D). Here, DrRR binding to DrBphP could not be detected in condition C. However, once DrRR is present in condition D, the DrBphP dimer peak is shifted and the oligomer peak disappeared, which points for DrBphP/DrRR interaction. In the case of AgP1, no changes in retention due to AtRR binding were detected. R = red light (655nm) illumination; FR = far-red light (785nm) illumination. Abbreviations: D = dark sample; R = red light (655 nm) illuminated sample.

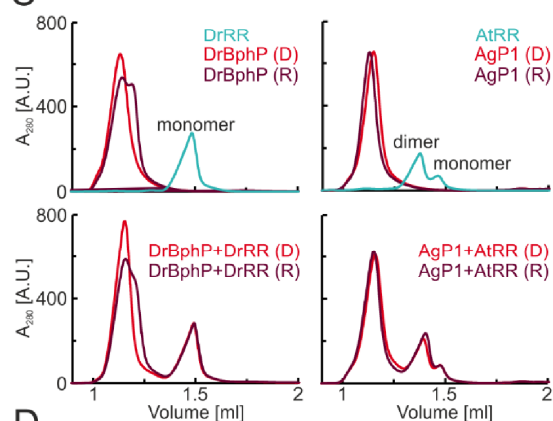
A

Sample	$A_{250/280}$	τ_1 (min)	A_1 (%)	τ_2 (min)	A_2 (%)	τ_3 (min)	A_3 (%)
DrBphP	1.25	0.8	18	18	24	480	59
DrBphP+DrRR	1.16	0.7	19	19	34	140	48
AgP1	1.31	2.3	18	13	49	240	33
AgP1+AtRR	1.40	2.2	12	13	48	250	40
Chimera	1.34	1.2	6	26	42	390	52
Chimera+AtRR	1.31	2.0	7	27	39	330	54

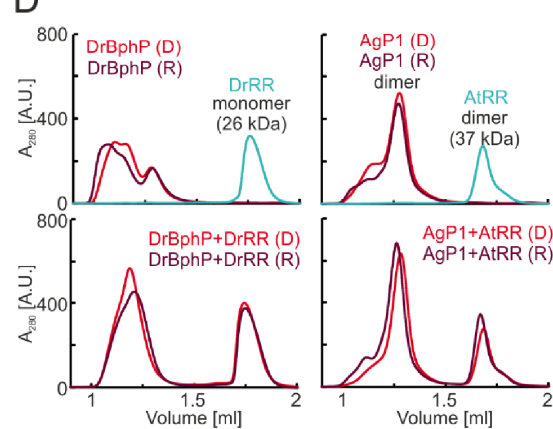
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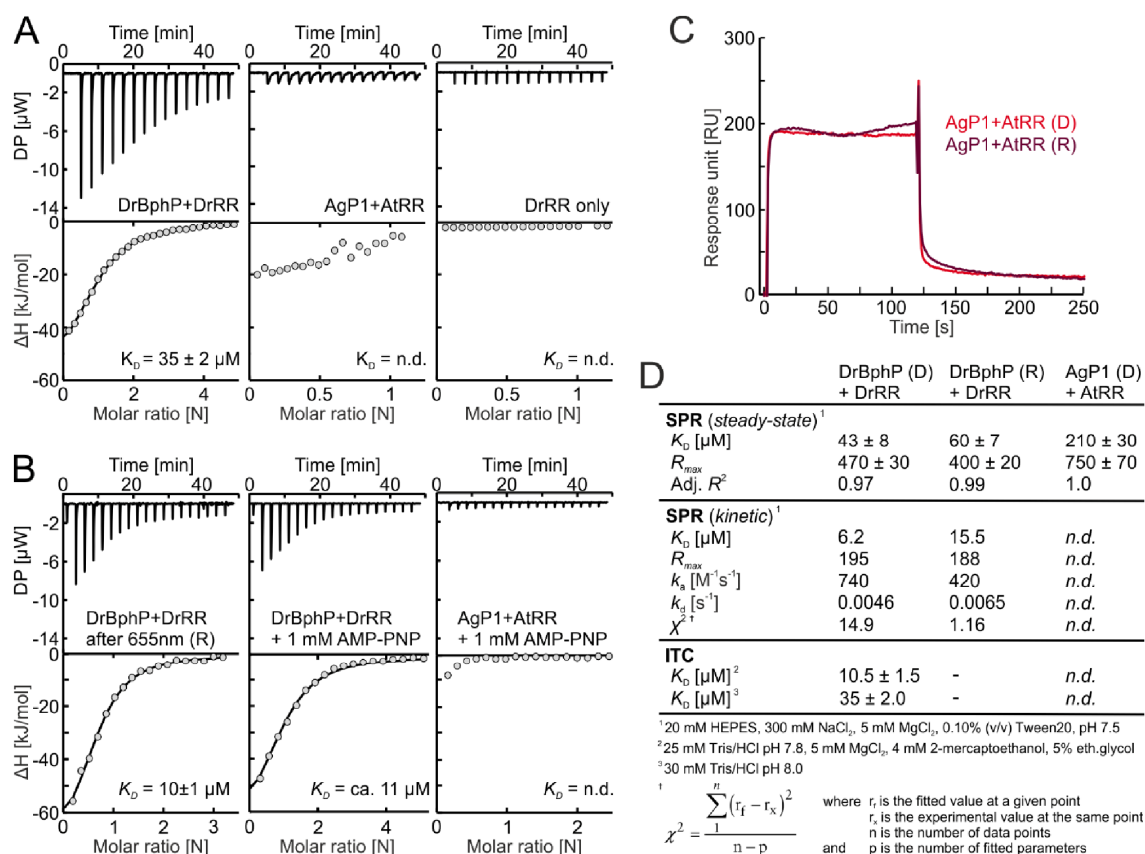
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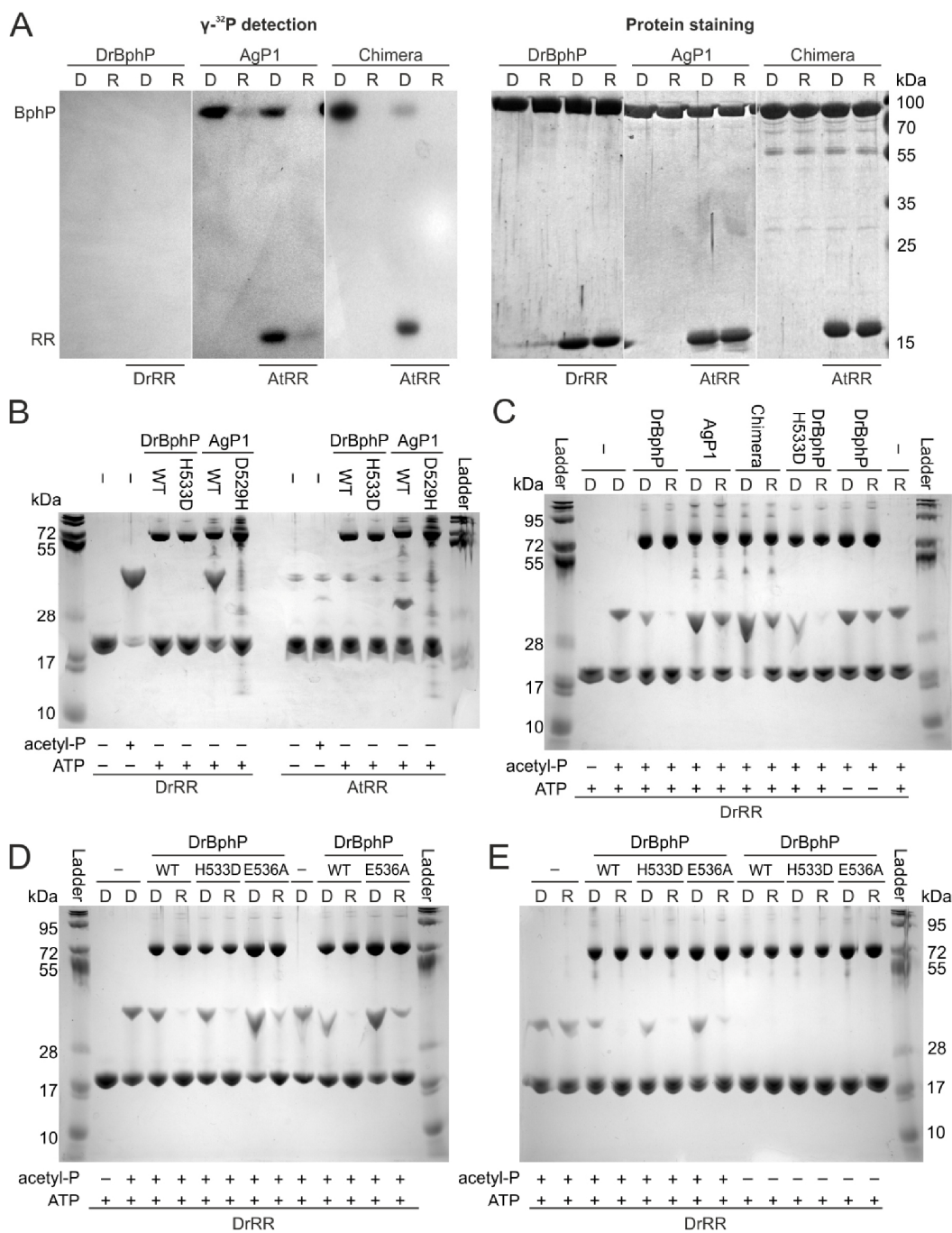
D



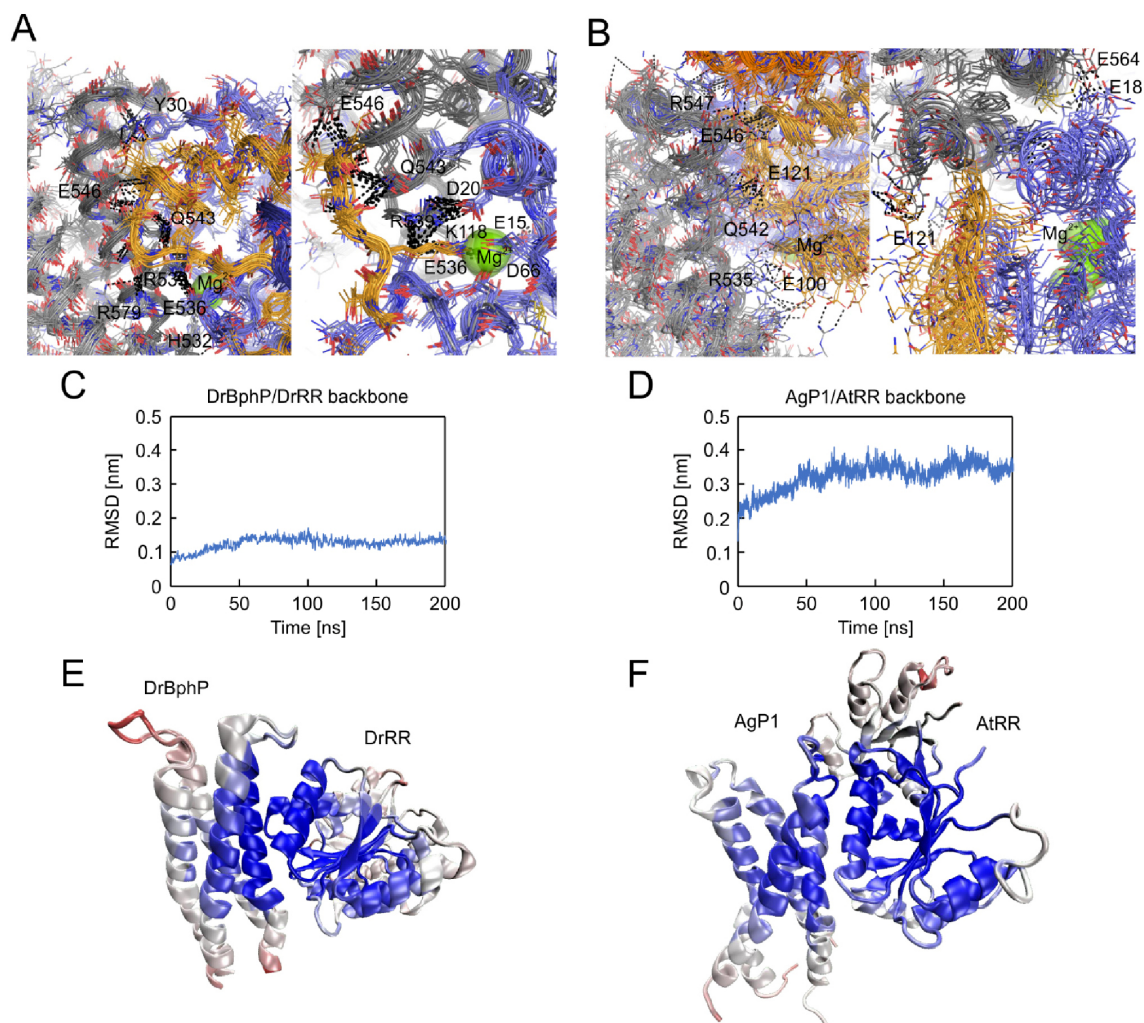
Supporting Figure S2. Additional measurements with isothermal calorimetry (ITC). **A.** DrRR binding to DrBphP measured in (30 mM Tris/HCl pH 8.0). DrBphP/DrRR interaction occurs with K_D of $35 \pm 2 \mu\text{M}$, and no AgP1/AtRR interaction was detected. **B.** DrBphP/DrRR interaction in buffer (50 mM Tris/HCl pH 7.8, 10 mM MgCl_2 , 8 mM 2-mercaptoethanol, 10% ethylene glycol) was unaffected by the red (655 nm) pre-illumination. This supports the result from SPR (Figure 2A) that illumination would not significantly affect the affinity. However, the ITC measurements have long pre-equilibration time (10 min) and slow acquisition time (~ 50 min), which leads to significantly reduced and constantly diminishing Pfr phytochrome population during the measurement. DrBphP/DrRR interaction and AgP1/AtRR interactions are unaffected by addition of 1 mM AMP-PNP in the buffer. **C.** Surface plasmon resonance (SPR) measurements of AgP1/AtRR interaction pair in dark (D) or after red light illumination (R). The measurement was conducted as in Figure 2A by applying AgP1 (100 μM) to AtRR-coupled sensor surface. **D.** Table summarizing the results from SPR and ITC analyses, along with additional fitting parameters. Adjacent R^2 -values indicate an agreement for the steady-state fits, with the best agreement approaching a value of 1.0. The χ^2 values close to 10 indicate a good kinetic K_D estimation in the case on DrBphP/DrRR interaction. R_{max} values give information on the maximal SPR response in saturating protein concentrations.



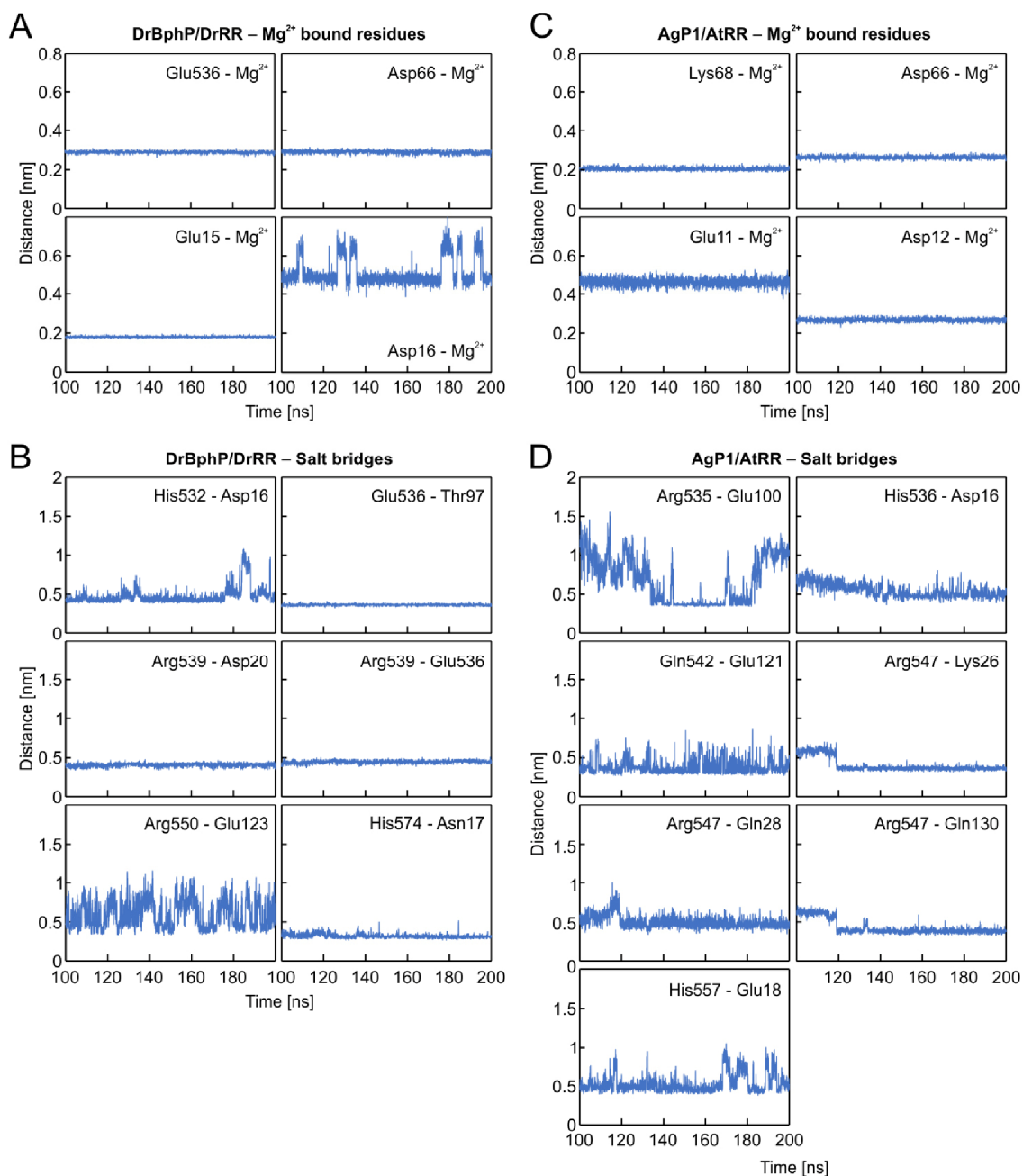
Supporting Figure S3. Full gels for radiolabeled and Phos-tag assays. **A.** Full gels from the radiolabeled ATP assay shown in the Figure 3A in the main text. **B.** Full gel of the kinase assay shown in Figure 3B. **C.** Full gel of the results shown in Figure 3C. The gel also shows that the phosphatase activity of DrBphP is stalled when ATP was excluded from the reaction. **D.** Full gel of the results shown in Figure 3D. **E.** Full gel of the results shown in Figure 3E. As seen in gels B and D, red light did not affect the stability of the free phospho-DrRR. The phytochrome samples are indicated above the gels. The response regulator, its treatment with acetyl phosphate treatment, and the inclusion of ATP are indicated below each gel. All measurements have been repeated at least three times, and not all repeats have been shown here.



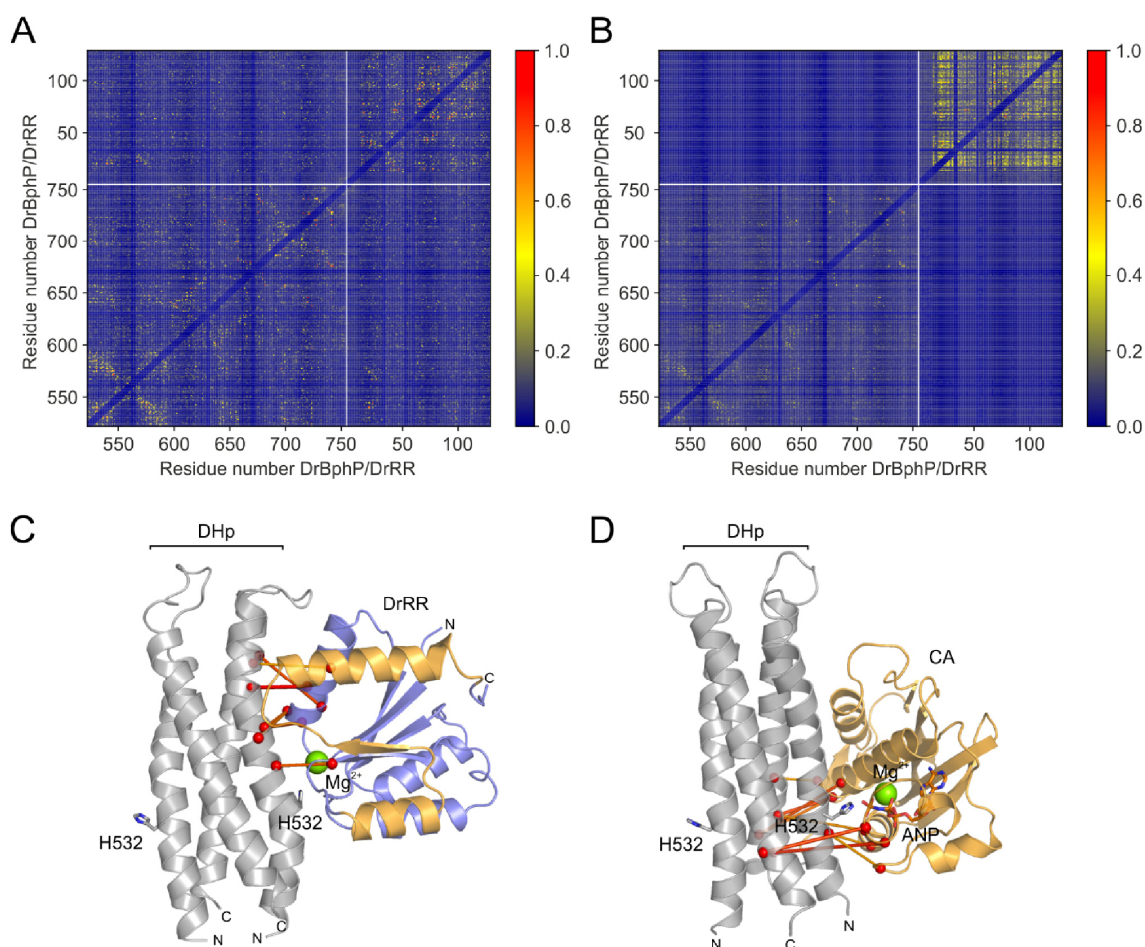
Supporting Figure S4. Flexibility of the HK/RR complexes in AgP1 and DrBphP. **A–B.** Relaxed solvated models of complexes DrBphP/DrRR (A) and AgP1/AtRR (B). 11 representative models are shown after global structural alignment by using PyMOL Molecular Graphics System version 2.3.3 (Schrödinger, LLC). Some of the polar interactions and salt bridges have been indicated. Molecules have been colored as in Figure 5 of the main text. **C–D.** RMSD of the backbone atoms along the 200 ns MD trajectory of DrBphP/DrRR complex (C) and AgP1/AtRR complex (D). **E–F.** Color maps of the amino acids heavy atoms root-mean square fluctuations (RMSF) for DrBphP/DrRR complex (E) and AgP1/AtRR complex (F). Blue color denotes rigid regions, red color denotes more flexible regions.



Supporting Figure S5. Distance plots of the structural parameters in HK/RR complexes. Each individual plot represents distance between the head groups of the residues at 100–200 ns in the MD trajectory after the initial 100 ns equilibration. **A.** Distances between Mg^{2+} ion and head groups of bound residues in DrBphP/DrRR complex. **B.** Salt bridges between DrRR and DrBphP proteins. **C.** Distances between Mg^{2+} ion and head groups of bound residues in AgP1/AtRR complex. **D.** Salt bridges between AtRR and AgP1 proteins.



Supporting Figure S6. Covariance analysis of in the DHp/RR and DHp/CA interfaces. **A.** Covariance matrix of the tandem BphP-RR sequences. Blue color indicates low and red high pairwise covariance. **B.** Covariance matrix of scrambled sequences. Here, little covariance between the HK and RR sequence segments is visible, thus verifying the specificity of the results shown in A. **C–D.** Strong covariances between residues in the DHp helices and their cognate response regulators (RR), and a catalytic ATP-binding (CA) domains. The same models are shown as in Figure 5A and Figure 6A. The extent of the covariance is color-coded in such a way that the red lines indicate high covariance and yellow lines intermediate covariance. Only the covariance lines above a cutoff value of 0.6 are shown.



Supporting Figure S7. Sequence variation of the key catalytic residues in histidine kinases. **A.** Sequence logo showing the conservation of selected residues in the HisKA occurrences. Two occasions have been shown: One where the H+1 position in the DHp helix is occupied by a histidine (His533 in DrBphP), another where H+1 site is not restricted to as a histidine. The sequence logo in the former occasion were plotted from 192 sequences, which resulted noisier graph than in the latter occasion. In the case where H+1 is histidine, the conservation of the N-box Asn in the CA domain is lost (H648 in DrBphP). However, the occurrence of Glu in H+4 position as well as Arg in position H+7 is increased in these cases. The key residues are shaded in yellow. **B.** Sequence alignment of representative bacteriophytochromes. Below the alignment are shown the key DrBphP residues discussed in the main text, along with consensus sequence of HisKA, HWE_HK, and phosphatase.

Alignment shows that the bacteriophytochromes above Cph1, and Cph1 itself, likely belong to HisKA family. Although DrBphP resembles other HisKA proteins, it misses a few functionally important residues. AgP1 has all the key residues required for HisKA activity. AgP2 and the phytochromes below it belong to HWE_HK proteins with a characteristic arginine residue in its H+1 position and a glutamate in the N-box of the CA domain. As for phosphatase activity, a threonine, asparagine or glutamate in H+4 position indicate that all selected phytochromes may also act as phosphatases. As a sole exception, the phosphatase activity of AgP1 may be hindered due to an alanine in this position. In conclusion, most phytochrome sequences have features that enable both kinase and phosphatase activity. Notable exceptions for this are AgP1 and DrBphP.

Full-length sequences were aligned in Jalview 2.11.1.0 (Waterhouse et al., 2009) using ClustalO (Sievers and Higgins, 2018; Sievers et al., 2011) with standard settings. The amino acid conservation was visualized with Boxshade version 3.21. Uniprot accession numbers in the same order as in the alignment: DrBphP (*Deinococcus radiodurans* - Q9RZA4), AgP1 (*Agrobacterium fabrum* - Q7CY45), SaBphP1 (*Stigmatella aurantiaca* - Q097N3), SaBphP2 (*Stigmatella aurantiaca* - Q09E27), RpBphP1 (*Rhodopseudomonas palustris* - Q6N5G3), RpBphP3 (*Rhodopseudomonas palustris* - Q6N5G2), PsBphP (*Pseudomonas syringae* - Q885D3), PaBphP (*Pseudomonas aeruginosa* - Q9HWR3), MaBphP (*Microcystis aeruginosa* - B0JT05), Cph1 (*Synechocystis* sp. PCC 6803 - Q55168), AgP2 (*Agrobacterium fabrum* - A9CI81), AvBphP (*Agrobacterium vitis* - B9K3G4), RiBphP (*Rhizobium leguminosarum* - Q1MCX7), ReBphP (*Rhizobium etli* - B3PX96), AcBphP (*Azorhizobium caulinodans* - A8HU76), RpBphP5 (*Rhodopseudomonas palustris* - Q6NB40), BjBphP (*Bradyrhizobium japonicum* - A0A023X9Y5).

6 Publikationsliste

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Golonka, D.; Kuznetsova, V.; Ihalainen, J. A.; Möglich. A. *Arabidopsis thaliana* phytochrome B incorporates biliverdin and undergoes light-dependent interactions. (not submitted)

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Multamäki, E.; Nanekar, R.; Morozov, D.; Lievonen, T.; Golonka, D.; Wahlgren, W. Y.; Stucki-Buchli, B.; Rossi, J.; Hytönen, V. P.; Westenhoff, S.; Ihalainen, J. A.; Möglich, A.; Takala, H. Illuminating a phytochrome paradigm – a light-activated phosphatase in two-component signaling uncovered. *Cell* (submitted)

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8 (Eidesstattliche) Versicherungen und Erklärungen

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