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Supplementary information

Plant phytochrome B is an asymmetric dimer with unique signalling potential

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Supplemental Information Guide

Plant Phytochromes are Asymmetric Dimers with Unique Signaling Potential

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Online Methods

Purification of Arabidopsis PhyB. The full-length PhyB polypeptide from the *A. thaliana* Col-0 ecotype (residues 1-1172) bearing an N-terminal 6His-TEV tag was expressed and assembled recombinantly with PΦB in *Escherichia coli* upon prior induction of the heme oxygenase and the HY2 PΦB synthase enzymes needed to convert heme to PΦB[1,2]. Various truncations and site-directed mutants were generated by Gibson assembly[3] and/or by QuikChange (Stratagene) with appropriate oligonucleotide primers, and were verified as correct by DNA sequencing of the full coding regions. The modulator loop was truncated by replacing residues 782-790 (NIQGDYKAI) with type-I (NPDG) and type-II β turns (NPGR) as guided by Wilmont and Thornton[4]. The biliproteins were purified at 4°C by sequential affinity chromatography with nickel-nitrilotriacetic acid beads (Qiagen) and anion-exchange FPLC with a Mono-Q 4.6/100 PE column (Cytiva)[1]. To ensure retention of the NTE, the preparations were subjected to an additional chromatography step with a Ni^{2+}-charged HiTrap IMAC HP column. The 6His-TEV tag was then removed by digestion with TEV protease followed by a second HiTrap IMAC HP step that removed the protease and high-affinity contaminants[1]. Prior to cryo-EM, the samples were further purified with a Superdex 6 Increase 10/300 SEC column in 75 mM KCl, 20 mM HEPES-KOH (pH 7.8), 10 mM 2-mercaptoethanol, and 1 mM Na_2EDTA. The integrity of the final PhyB preparations was confirmed by UV-vis absorption spectra, and by SDS-PAGE followed by staining of the gels either for protein with Coomassie Blue or for PΦB by zinc-induced fluorescence under UV light[1] (Extended Data Fig. 1a,b).

Cryo-EM grids preparation and data collection. Three μl aliquots of PhyB (0.3 mg/ml) were applied to freshly glow-discharged Quantifoil R2/1 300 mesh copper grids and then blotted with a piece of Whatman 595 filter paper with the blot force set to 2 and blot time set to 3 sec. The blotted grids were plunge frozen into liquid ethane cooled by liquid nitrogen in a FEI Vitrobot (Mark IV), with the chamber temperature set to 6°C and the chamber humidity set to 95%, and then stored in liquid nitrogen. Cryo-EM images were recorded with a Gatan K2 camera at a magnification corresponding to a pixel size of 1.029 Å at the specimen level. The dataset was collected automatically using the EPU-2.3 software in a Titan Krios microscope (ThermoFisher

Scientific) operated at 300 kV. Forty-frame movies were recorded with a dose rate of 7 electrons•pixel^{-1}•sec^{-1} and an exposure time of 8 sec.

Image processing. A total of 6,153 movies were acquired, which were motion corrected with MotionCor2-1.1.0[5], and their contrast transfer function (CTF) effects corrected in CtfFind-4.1.8[6]. After visual inspection of particle distribution, contrast, and Thon ring quality, 4,573 micrographs were retained for further processing. Using RELION-2[7], we manually picked approximately 1,000 particles to generate several 2D averages, which were used as templates for the subsequent automatic particle picking. In total, 902,965 particles were chosen and subjected to 2D classification, of which 528,057 particles belonging to 2D classes with clear structural features were retained for subsequent 3D classification. Based on 3D classification, 286,021 particles belonging to two high quality 3D classes were selected for further 3D reconstruction and refinement, leading to a 3D map at 3.4-Å average resolution (Extended Data Fig. 1c). This map showed a well-ordered core (nPAS, GAF, PHY, PAS2 and HKRD regions), and a less-ordered PAS1 domain. To improve the core density, these particles were subjected to signal subtraction and masked 3D classification with a soft mask that included the nPAS, GAF, PHY, PAS2 and HKRD regions. Two classes contained 154,901 particles with the best structural features. These particles were selected and further refined to 3.3-Å resolution by 3D refinement, CTF refinement, and Bayesian polishing in RELION-3.0[8]. To improve the PAS1 domain density, we down-sampled the particle images by a factor of 2 (to 2.058 Å/pixel) and first subjected the particles to a consensus refinement. We then performed 3D variability analysis in cryoSPARC-3.2 with a resolution filter (cutoff) set to 10 Å[9]. A set of 20 3D maps were obtained in the dynamic 3D video; the one with the best PAS1 domain EM density is shown in Fig. 1d and Extended Data Fig. 1c.

Model building, refinement, and validation. The crystallographic structure of the *A. thaliana* PhyB PSM (residues 90-624; PDB: 4OUR[10]) was fitted into the EM map using UCSF Chimera1.15[11]. The restraint file for PΦB refinement was generated from ligand O6E extracted from the crystallographic structure of the PAS-GAF bidomain from *S. bicolor* PhyB (PDB: 6TC5[12]). The remaining PAS2 and HKRD domains were manually built in COOT version 0.9.5[13]. The completed model was refined iteratively by real-space refinement with PHENIX version 1.19.2-4158-00[14] and manual adjustment in COOT. The final atomic model was validated using MolProbity version 4.5[15]. To avoid overfitting, we verified the refined model by correlation with the final 3D map and the two half-maps (half-1 and half-2) through three Fourier shell correlation (FSC) curves analyzing model vs. final map, model vs. half1 map (FSC_{work}), and model vs half2 map (FSC_{free}) (Extended Data Fig. 1h). The PSM-PAS2 surface was estimated by Proteins,

Interfaces, Structures and Assemblies (PISA) in PDBe (https://www.ebi.ac.uk). Structural figures were prepared in UCSF Chimera[11], UCSF ChimeraX-1.2.5[16], and PyMOL (https://pymol.org/2/). 3D predictions of the three PAS domains were created by TrRosetta[17]. Amino acid sequence conservation among seed plant Phys were generated in Clustal Omega (http:/www.clustal.org) from 177 sequences representing the PhyA, PhyB, PhyC and PhyE isoforms and displayed by hand in Adobe Illustrator[1].

Biochemical analysis of PhyB mutants. UV-vis absorption spectra of dark-adapted Pr and red light-irradiated Pfr states were acquired with a Cary60 spectrophotometer (Agilent) equipped with temperature-controlled cuvettes, using preparations exchanged into standard assay buffer (50 mM HEPES-HCl (pH 7.8 at the experimental temperature), 150 mM KCl, 100 mM arginine, 1 mM Na_2EDTA, and 10 mM 2-mercaptoethanol) previously designed for the long-term solution stability of plant Phys[1]. Spectral change ratios (SCR) were derived from the Pr-minus-Pfr absorbance difference spectra (Δabsorbance of the Q band maximum as Pr/Δabsorption of the Q-band minimum as Pfr). Pfr → Pr thermal reversion was measured at 25°C or 21°C in darkness as described[1] after driving the Pfr levels to steady state with a 630-nm LED. Reversion kinetics were monitored spectrophotometrically from 500-900 nm; the corresponding fits were calculated from wavelengths ranging from 640-750 nm. Data presented in the Figures were normalized using calculated rate constants (Extended Data Table 3), amplitudes, and baselines at 725 nm. All spectroscopy data were collected using Cary WinUV software. Data were extracted, processed, and fit using R Studio (http://www.r-project.org).

Protease accessibility was assayed by incubating 200 ng/μL of PhyB with various concentrations (0-20 ng/μL) of either glutamyl endopeptidase (GluC) or chymotrypsin (Sigma) for 20 min at 25°C. The fragments were then digested to completion with trypsin and analyzed by tandem MS with a ThermoFischer Q-Exactive-Plus mass spectrometer after peptide separation by reverse-phase nano-HPLC with a 25-cm analytical C18 resin column (Acclaim PepMap RSLC; Thermo Fisher Scientific) and a 5 to 95% acetonitrile gradient in 0.1% formic acid. Peptides ending in GluC (Glu and Asp) or chymotrypsin (Phe, Tyr, Trp and Leu) cleavage sites were quantified from the MS1 spectra using Proteome Discoverer (version 2.0.0.802; ThermoFisher Scientific).

Autophosphorylation assays were performed in kinase assay buffer (25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM EDTA and 4 mM 2-mercaptoethanol) with 1 μg of full-length Arabidopsis PhyB or the N982, N624 truncations, and full-length BphP from *Pseudomonas syringae*[18] in either their dark-adapted Pr state or upon saturating red light irradiation (mostly Pfr).

The biliproteins were synthesized and assembled in *E. coli* with their native chromophores (PΦB for *At*PhyB or biliverdin for *Ps*BphP), and purified as described[8,17]. Reactions were initiated by adding 150 µM ATP containing 10 µCi of [γ-^{32}P]-ATP followed by incubation for 2 hr at 25°C. Reactions were quenched with SDS-PAGE sample buffer, heated to 100°C, and subjected to SDS-PAGE, followed by exposure of the gels to imaging plates (GE Health Care BioSciences). Autophosphorylation was quantified with an Amersham Typhoon phosphorimager[18].

Estimation of PhyB size by SEC and data fitting. The apparent sizes of PhyB and various mutants were determined by SEC using an AKTA FPLC (GE Health Sciences) equipped with a Superdex 200 Increase 10/300 GL column (Cytiva) equilibrated at 22°C in standard assay buffer that substituted 10 mM 2-mercaptoethanol with 1 mM tris(2-carboxyethyle)phosphine; 250 µL samples were eluted at a 0.5 ml•min^{-1} flow rate. Sample concentrations were estimated by absorbance at 280 nm using ProtParam[19]. The column was calibrated for molecular mass using BioRad molecular weight markers and blue dextran. FPLC profiles were collected using the Unicorn software (Cytiva).

To estimate relative affinities for subunit assembly, the following equation was derived. Equilibrium between monomers and dimers were expressed through the law of mass action, $K_2 = C_d C_m^{-2}$ (Equation 1), where K_2 is the equilibrium assembly constant and C_d and C_m are the molar concentrations of dimers and monomers, respectively. Total subunit concentration was $C_{tot} = 2C_d + C_m$ (Equation 2). From equations 1 and 2, $C_d = 0.125(1 + 4K_2C_{tot} - (1 + 8K_2C_{tot})^{0.5})K_2^{-1}$ (Equation 3). Equation 3 can be equated to mass average dimer fraction by $f_d = (MW_i – MW_m)(MW_d – MW_m)^{-1} = 2C_dC_{tot}^{-1}$ (Equation 4), where MW_i, MW_d and MW_m are the estimated molecular weights for the sample, dimers and monomers, respectively. Combining Equations 3 and 4 and solving for the sample molecular weight yields $MW_i = MW_m + 0.25(MW_d – MW_m)(1 + 4K_2C_{tot} - (1 + 8K_2C_{tot})^{0.5})K_2^{-1}C_{tot}^{-1}$ (Equation 5). For the N908 and N928 fragments, MW_m was estimated using the average values from the F420E and I855E mutants. Other coefficients were estimated by R Studio. We assumed that MW_d was identical for wild-type and the L427A version of N908, and for N928; thus, those values were fit globally, while K_2 was allowed to vary by sample. For the N982 fragment, all variables were allowed to float.

Concentration dependence of PhyB(N908) on thermal reversion rate. Thermal reversion rates for the N908 fragment at a wide range of concentrations were measured as described above. For measurements at low concentrations, 4 and 15-cm pathlength chambers were constructed to increase absorption, and the rate constant (k) was calculated with a single exponential. Solution

conditions from the SEC experiments were used, except that the temperature was 21°C. The assembly constant (K_2) was calculated with the following equation, $k_{obs} = k_{min} + (k_{max} – k_{min}) • (C_d • (C_m + C_d)^{-1})$, where k_{obs}, k_{min} and k_{max} are the observed, minimal and maximal thermal reversion rate constants, concentration of dimers is Equation 3, and concentration of monomers as derived from Equations 1 and 2 ($C_m = 0.25(-1 + (1 + 8K_2C_{tot})^{0.5}) •K^{-1}$). The dissociation constant (K_D) is the inverse of K_2. k_{min} was fixed using the observed rate constant for the F420E mutant of the N908 fragment. Fits were conducted with Excel (Microsoft).

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Legends for Supplementals Videos

Supplemental Video 1. Cryo-EM structure of Arabidopsis PhyB. A surface rendering of the 3D map, fitted with the atomic model, is shown rotating around a horizontal axis by 360°, followed by rotation around a vertical axis by 360°. Then the density fades away and the atomic model rotates as before. The domains are individually colored as in Fig. 1. PΦB is shown as spheres.

Supplemental Video 2. Morph between protomers A and B of Arabidopsis PhyB. Protomer A and B were superposed based on the B and C rings of PΦB. Transitioning from protomer A and B, minor conformational changes radiate and propagate from PΦB to the bilin-binding pocket, then through the GAF domain, and finally spreading to the whole molecule. PΦB is colored in purple for protomer A and in cyan for protomer B. The domains are individually colored as in Fig. 1.

Supplementary Figure 1| Raw SDS-PAGE gels and autoradiographic images presented in this study. Images correspond to those found in Extended Data Figures 1a, 10b and 10c, and 13a and 13b.

Supplementary Table 1| Source data for the graphs in Figures 3b-d.

Supplementary Table 2| Source data for the graphs in Figures 4d and 4e.