Research Article

A Spectroscopy-based Methodology for Rapid Screening and Characterization of Phytochrome Photochemistry in Search of Pfr-favored Variants

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ABSTRACT

Phytochromes are photosensitive proteins with a covalently bound open-chain chromophore that can switch between two principal states: red light absorbing Pr and far-red light absorbing Pfr. Our group has previously shown that the bacteriophytochrome from Xanthomonas campestris pv. campestris (XccBphP) is a bathy-like phytochrome that uses biliverdin IXα as a co-factor and is involved in bacterial virulence. To date, the XccBphP crystal structure could only be solved in the Pr state, while the structure of its Pfr state remains elusive. The aims of this work were to develop an efficient screening methodology for the rapid characterization and to identify XccBphP variants that favor the Pfr form. The screening approach developed here consists in analyzing the UV-Vis absorption behavior of clarified crude extracts containing recombinant phytochromes. This strategy has allowed us to quickly explore over a hundred XccBphP variants, characterize multiple variants and identify Pfr-favored candidates. The high-quality data obtained enabled not only a qualitative, but also a quantitative characterization of their photochemistry. This method could be easily adapted to other phytochromes or other photoreceptor families.

INTRODUCTION

Phytochromes are photosensitive proteins with a covalently bound open-chain tetrapyrrole chromophore that can switch between two principal states: red light absorbing (Pr) and far-red light absorbing (Pfr). Typically, phytochromes present an N-terminal photosensory module composed of the PAS2-GAF-PHY triad domain and an output module located toward the C-terminus. The output module can present a variety of functions which may include multiple or single enzymatic or nonenzymatic domains (3,5,6). Phytochromes can be classified as “canonical” or “bathy” depending on whether they decay in the dark (dark conversion) toward a Pr or Pfr thermally stable state, respectively (2,7–9). Since the pioneering works using purified phytochromes from natural plant sources (10–12), recombinant phytochromes have paved the way to more rapid biochemical, photochemical and structural studies (13–16).

In the last few years, our group has studied the structure, photochemistry and biological function of the bacteriophytochrome from the phytopathogen Xanthomonas campestris pv. campestris (XccBphP) (17–19). XccBphP has been demonstrated to be involved in bacterial virulence. As typical bacterial phytochromes, XccBphP covalently binds biliverdin IXα (BV) as its chromophore through the auto-catalytic bilin-lyase activity of the GAF domain, giving rise to the photosensory properties by BV-photoisomerization (20–24). When XccBphP is irradiated with far-red light, it photoconverts to the Pr form, while red light irradiation gives rise to a mixture of Pr and Pfr (~2:1 with a 630-nm light source). In XccBphP, dark conversion leads to a further enrichment of Pfr, reaching a Pfr:Pr proportion of ~6:1 at equilibrium; we therefore have classified XccBphP as a bathy-like phytochrome (17). We have previously solved the crystal structure of the full-length XccBphP in the Pr state (PDB code: 5AKP) (17), which harbors the PAS2-GAF-PHY domain triad as its photosensory module and a PAS9 output domain (Fig. 1). However, the Pfr structure remains elusive. In order to generate a more complete picture of the structural changes that occur during the Pr-Pfr transition, the structure of its Pfr is needed.

The aims of this work are the development of an efficient screening methodology for the rapid characterization of phytochrome variants and the identification of variants in XccBphP that favor the Pfr form. These variants could become valuable tools in our attempt to obtain crystal structures of XccBphP in the Pfr conformation. Here, we present a methodology for a rapid spectroscopic characterization of phytochrome variants. The screening approach consists of analyzing the UV-Vis absorption behavior of clarified crude extracts from Escherichia coli overexpressing recombinant proteins in microplates. This strategy has allowed us to (1) quickly explore over a hundred XccBphP variants, (2) characterize their photochemistry and (3) identify Pfr-favored candidates for future studies. This method could in principle be easily adapted and applied to other phytochromes or other photoreceptor families to find variants with altered photochemical behavior.
Amino acid frequency analysis. Sequences from the UniProtKB database were searched against the PHY domain HMM profile retrieved from Pfam database (https://pfam.xfam.org/), family PF00360) using the hmmssearch program in the EBI server (https://www.ebi.ac.uk/Tools/hmmer/) and default parameters (Input file: PHY hmm; Output file: UniProtKB_IDs.txt). The 15,193 positive UniProtKB AC/ID entries were mapped (clustering homologs to reduce redundancy) using the online mapping tool provided online by the EBI server (https://www.uniprot.org/uploadlists/) to reduce the number of UniProtKB redundant and highly similar sequences. The 1190 resulting mapped UniRef50 entries were downloaded in a FASTA format file (Input file: UniRef50.fasta; Output file: UniRef50_IDs.fasta). The UniRef50 FASTA sequences were scanned locally against PAS2, GAF and PHY domain HMM profiles retrieved from Pfam database (families PF08446, PF01500 and PF00360, respectively) using HMMER 3.2 (http://hmmer.org/) (Input files: UniRef50.fasta, PAS2 hmm, GAF hmm, PHY hmm; Output file: TableDom_UniRef50.out). 750 sequences that exhibit a PAS2-GAF-PHY domain architecture were identified and their respective GAF domain sequences extracted in FASTA format file (Input files: TableDom_UniRef50.out, UniRef50 fasta; Output file: BphP_UniRef50_GAF.fasta). A multiple sequence alignment (MSA) was performed using Clustal O program in the EBI server (https://www.ebi.ac.uk/Tools/clustalo/) and default parameters of 751 GAF domains retrieved from UniRef50 and XC_4241 (Xcc_BphP) sequences (Input file: BphP_UniRef50_GAF.fasta; Output file: clustalo-GAF_UniRef50.clustal_num). The Xcc_BphP L193 homolog residue at the MSA was analyzed for amino acid frequency.

Protein sequences were analyzed using custom MATLAB (version 2015a) scripts. The results are shown in Table S1, and all files mentioned in the above paragraph are provided as Supporting Information.

Figure 1. Design of XccBphP variants. Selected positions for mutagenesis analysis mapped on the XccBphP Pr structure. The overall crystal structure (PDB code: 5AKP) is shown on the right. Each domain, PAS2, GAF, PHY (photosensory module) and PAS9 (output module), is shown with a different color. The tongue region in the PHY domain is indicated with a dashed box. The biliverdin molecule (BV) is shown as sticks. In the inset, key positions selected for mutagenesis are represented as sticks and atoms are colored according to their type.

MATERIALS AND METHODS

Protein overexpression and purification. Protein-enriched cell lysates used in the screening were obtained as follows. Escherichia coli BL21 (DE3)pLysS cells transformed with pET-24a-XccBphP constructs were grown overnight in 50 ml of LB medium with 25 μg ml−1 kanamycin and 17 μg ml−1 chloramphenicol at 37°C in a 250 ml Erlenmeyer flask with agitation (220 rpm). Cultures were then diluted 100-fold, grown to an optical density at 600 nm of 0.6 and induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) final concentration. Cultures were further incubated for 16 h at 20°C with agitation (220 rpm) and then centrifuged at 9800 g for 15 min at 4°C. Bacterial pellets were resuspended in one tenth of their original culture volume and sonicated in 50 mM Tris-HCl pH 7.5, 0.25 M NaCl. Lysates were then clarified by two rounds of centrifugation at 21 000 g for 30 min at 4°C, and the supernatant fractions obtained were used as apoprotein sources. Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to assay protein levels (Figure S2).

The purification of wild-type (WT) XccBphP holoprotein was performed as previously described (17,25). Holoproteins were produced by incubation of purified apoproteins for 1 h in absence of light at room temperature. Further purification steps were performed under dim green-filter light (band-pass filter maximum at 525 nm), minimizing XccBphP excitation.

UV-Vis spectra measurements. The screening measurements were performed in a Thermo Scientific Multiskan GO Microplate Spectrophotometer using Thermo Scientific Multiskan GO Microplate Spectrophotometer 96-multiwell plates (Thermo Fisher Scientific). This equipment allows the simultaneous measurement of UV-Vis spectra in 96-well plates in thermostatic and dark conditions and with programmed sampling. The volume of cell lysate used for each well was 200 μl. After the addition of BV (Sigma-Aldrich, product number 30891), the supernatant fractions were incubated at 25°C in darkness for 20 min. As a reference, after a 5-min incubation with BV, the holoprotein was completely assembled (17). We chose the time of 20 min as a trade-off between maximizing the BV assembly and minimizing the total time that the clarified crude extracts are at nonrefrigerated temperatures to avoid denaturation/precipitation/proteolysis phenomena. For the 2-h experiments, the samples were then irradiated with red (625 nm) light at 0.2 μmol m−2 s−1 flux for 20 min and their UV-Vis spectra acquired at 0-, 1- and 2-h postirradiation between 240 and 850 nm in the dark. After the 2-h incubation in the dark, the samples were irradiated with far-red (733 nm) light at 0.6 μmol m−2 s−1 for 7 min and final UV-Vis spectra were acquired. For the ~6 h experiment, the samples from the G454X library were irradiated with far-red (733 nm) light at 0.6 μmol m−2 s−1 flux for 7 min and their UV-Vis spectra acquired at 0 to 970 min postirradiation between 240 and 850 nm in the dark.
sampling times were the following: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 80, 100, 130, 190, 250, 310, 370, 490, 610, 730, 850 and 970 min. For purified WT, UV-Vis absorption spectra were collected for 17 times at 20.0 ± 0.2°C on a Cary60 UV-Vis spectrometer (Agilent) with an installed temperature control Peltier module. The sample was illuminated with far-red light at a fluence of 0.6 μmol m⁻² s⁻¹ for 7 min prior to the measurements in the dark. The protein concentration was 14 μM in 50 mM Tris pH 7.5, 0.25 M NaCl.

UV-Vis spectra analysis. All the steps of the analysis were performed using custom made pipelines developed in MATLAB software (version 2015b). For each G454X variant, the UV-Vis datasets consisted of 25 spectra acquired from 0 to 970 min. For visualization for each variant, the minimum values were subtracted and then each spectrum was normalized to the maximum value in the dataset in the 540-850-nm range. As an observation, three variants (G454R, G454H and G454D) seem to present an incomplete BV assembly by the time the dark conversion begins. Due to the high background in the near UV and blue regions, the datasets were processed and trimmed to the 540–850-nm range. For each spectrum, a linear baseline fit (nonzero slope) was calculated and subtracted, then normalized to the highest value in the range. In each processed dataset, the spectrum that exhibited the highest Abs688nm/Abs752nm relation (corresponding to spectra close to time 0 min, immediately after far-red irradiation) was smoothed and selected to act as the corresponding pure-Pr spectral component (pure-Pr). The pure-Pfr spectral component (pure-Pfr) estimation was obtained by performing the subtraction of a multiple of pure-Pr from the last spectrum acquired in the dataset that best approximates to a straight line performing the subtraction of a multiple of pure-Pr from the last spectrum acquired in the dataset that best approximates to a straight line. The pure-Pr and pure-Pfr spectra for each variant were compared.

RESULTS

Design of XccBphP variants

We designed a series of variants in XccBphP based on (1) the spectroscopic behavior described in other phytochromes, (2) the analysis of the structure of XccBphP in the Pr state and (3) the analysis of amino acid conservation and frequency within the GAF domain of the phytochrome family (Table 1 and Fig. 1). The chosen homologous mutations reported to affect the photochemistry of other phytochromes comprise the substitutions D199A, V266S and L193 (various substitutions, detailed below) in the GAF domain (27–29), and S474E and G454E in the tongue region of the PHY domain (29). The double substitution L193C-F475C was designed to favor the Pfr conformation by crosslinking through a covalent disulfide bridge between the positions 193 and 475, which are distant in space in the Pr form and should, in principle, be facing each other in the Pfr state, as judged by the conformation of the homologous positions in the crystal structure of Pseudomonas aeruginosa BphP (27,28). Finally, a series of variants at the position 193 of XccBphP were chosen, according to their natural frequency in the homologous position within the GAF domain from the phytochrome family that contains the PAS2-GAF-PHY domain triad (Table S1). The photochemistry of all these variants mentioned above was characterized by a rapid screening method, which is the central theme of this article.

Additionally, based on previous works which have generated high-resolution crystal structures of other phytochromes (30–36), and reports that propose that the PHY domain plays a key role in modulating the Pr-Pfr photochemistry (6,37,38), two short constructs were designed composed of only the PAS2 and GAF domains (containing residues 1 to 313), namely PG-WT and PG-

A similar pipeline was applied to the purified wild-type where all of the steps mentioned above were applied with the exception of the UV-Vis spectrum trimming and linear baseline subtraction.

Each processed dataset is graphically shown in individual GIF files available in Supporting Information, overlaying individual pure-Pr and pure-Pfr spectral components, and their relative abundances at each time point.

Table 1. XccBphP variants used in this study.

| XccBphP variant | Position in XccBphP structure | Homolog mutation | Effect in homolog model | References |
|-----------------|-------------------------------|-----------------|-------------------------|------------|
| D199A           | GAF                           | PoBphP-D194A    | Locks Pr                | Yang et al. (28) |
| V266S           | GAF (BV contact in Pr)        | PoBphP-S261A    | Locks Pfr               | Yang et al. (28) |
| G454E           | Tongue in PHY                | ApPhyB-G564E    | Favors Pfr              | Burgie et al. (29) |
| S474E           | Tongue in PHY                | ApPhyB-S384E    | Favors Pr               | Burgie et al. (29) |
| L193I           | GAF, BV contact in the Pfr form | ApBphP-Q188L | Favors Pr               | Burgie et al. (31) |
| L193R           |                               |                 |                         |             |
| L193M           |                               |                 |                         |             |
| L193F           |                               |                 |                         |             |
| L193E           |                               |                 |                         |             |
| L193C-F475C     | GAF and tongue (PoBphP homolog positions) | - | - | Yang et al. (28) |
| PG-WT           | PAS2-GAF                      | DvBphP          | High-resolution structures | Wagner et al. (2008), Burgie et al. (29) |
| PG-D199A        |                               | DvBphP          | High-resolution structures |             |

\[ f(t) = A + B \cdot e^{c_1t} + D \cdot e^{c_2t} \] (1)
D199A. With the aim to use these constructs to obtain high-resolution structures, we decided to evaluate their photochemical behavior previous to crystallization and included them in the screening described below.

**Screening settings**

The method takes advantage of the fact that the *E. coli* clarified extracts do not considerably absorb light in the visible range. Briefly, it consists of overexpressing the XccBphP variants in *E. coli* BL21(DE3)pLysS, lysing the cell cultures, incubating in the dark with an appropriate amount of BV for holoprotein assembly and measuring the UV-Vis spectra (Fig. 2). The UV-Vis spectra of all the XccBphP variants cell lysates were acquired simultaneously in a microplate spectrophotometer.

The volume of cell lysate needed for the experiment is relatively small (200 μl), and for this reason we explored scaling down the volume of the XccBphP overexpressing cultures relative to our standard affinity tagging purification protocol. The initial idea was to perform this step in a culture type 96-well plates (2 ml per well). However, protein expression levels were below the sensitivity threshold for the screening. Then, we assayed gradually incrementing cultures volumes from 5 to 50 ml, using 50-ml falcon-type plastic tubes (5–10 ml cultures) and Erlenmeyer flasks (10–50 ml cultures) (Fig. S3, left panel). The minimal culture volume with appropriate protein expression levels selected was 25 ml in a 250-ml Erlenmeyer flask for WT. Regarding reproducibility, culture volumes of 50 ml in 250-ml Erlenmeyers proved to be the more effective ones for various variants tested; therefore, this condition was chosen for the screening.

Free BV presents a broad and low-intensity absorption peak around 660 nm and upon holoprotein assembly it gives rise to narrower and more intense peaks, namely the Q bands (25,39). If free BV concentration is relatively high, this broad peak can mask the holo-XccBphP signal due to spectra overlap. In order to optimize the holoprotein signal, we lowered the amount of BV by minimizing free BV signal while maintaining holoprotein signal as high and clean as possible (Fig. S3, right panel). The BV final concentration selected for the screening was 21.9 μM.

**Screening results**

After optimizing the screening conditions, the selected XccBphP variants mentioned above were assayed. The samples were incubated in the dark for at 0, 1 and 2 h after irradiation with red light from a LED source (625 nm). The red light, which generates a Pr:Pr mixture (ratio ~ 2:1 in WT), allows for the identification of either Pr or Pfr enrichment (17). Then, at the end of the incubation, the samples were irradiated with far-red light to photoconvert them to the Pr state (Figs. 3–4, panel A). All of them exhibited dark assembly between apoproteins and BV into holoproteins in the Pr state (Fig. 3B and Table 2).

From the comparison of the spectra after 1-min and 148-min postincubation, it can be observed that in most variants the amount of the Pr is increased, indicating that the BV assembly is partial after 1 min. Apparently, the rate of BV assembly varies dramatically among the variants (Fig. 3B). Additionally, all the variants present an altered photocycle compared to the wild-type (Fig. 4B and Table 2). Several mutations resulted in Pr-locked versions of XccBphP, including the truncated versions PG-WT and PG-D199A, comprising only PAS2 and GAF domains (Table 2). The substitution D199A also generated Pr-locked species (Table 2). This was not unexpected as the homologous mutation performed in PsaBphP resulted in a Pr-locked state (27).

This Pr-locking behavior can be explained by the elimination of
Figure 3. Screening results: BV assembly of XccBphP variants. (A) Scheme of the temporal sequence of light treatment and measurements. The highlighted measurements correspond to the ones used for the qualitative analysis of BV assembly, shown in panel B. (B) UV-Vis spectra from clarified apoprotein extracts after the addition of BV in the dark. The black traces correspond to the spectra collected 1 min after the addition of BV (#1), while the purple traces correspond to the measurements at 148 min (#5), after far-red illumination.
the stabilizing contact between D199 and BV ring-D in the Pfr form (PDB entry 3C2W) (27). Interestingly, the substitution in the XccBphP-V266S homologous position for PdbPbP (S261A) results in a strong stabilization of its Pfr state due to the elimination of the interaction between the Ser residue and the propionate group from BV ring-C established in the Pr conformation (PDB entry 3G6O) (28). Following the same rationale, the addition of group from BV ring-C established in the Pr conformation (PDB entry 3C2W) (27). Interestingly, the substitution in the stabilizing contact between D199 and BV ring-D in the Pfr form (PDB entry 3G6O) (28). Following the same rationale, the addition of group from BV ring-C established in the Pr conformation (PDB entry 3C2W) (27).

### Table 2. Results of the screening of XccBphP variants.

| XccBphP variant | Dark assembly of BV | Enrichment in darkness | Sensitivity to light | Rate |
|-----------------|---------------------|------------------------|---------------------|------|
| D199A           | Pr                  | Pr                     | Locked              | –    |
| V266S           | Pr                  | Pr                     | Unlocked            | Slow |
| G454E           | Pr                  | Pfr                    | Unlocked            | Fast |
| S474E           | Pr                  | Pr                     | Unlocked            | Fast |
| L193H           | Pr                  | Pr                     | Unlocked            | Fast |
| L193T           | Pr                  | Pr                     | Unlocked            | Slow |
| L193R           | Pr                  | Pr                     | Unlocked            | Slow |
| L193M           | Pr                  | Pfr                    | Unlocked            | Fast |
| L193F           | Pr                  | Pfr                    | Unlocked            | Slow |
| L193E           | Pr                  | Pr                     | Unlocked            | Fast |
| L193C-F475C     | Pr                  | Pr                     | Locked              | –    |
| F475C           | Pr                  | Pr                     | Locked              | –    |
| PG-WT           | Pr                  | Pr                     | Locked              | –    |
| PG-D199A        | Pr                  | Pr                     | Locked              | –    |

Variants insensitive and sensitive to light are named locked and unlocked, respectively. Variants showing or not showing significant changes in their relative absorbance at 688 and 752 nm after 1 h are referred to as fast and slow, respectively.

Strikingly, the L193C-F475C variant not only failed in locking XccBphP in Pfr as intended, but resulted in the exact opposite behavior, locking it in the Pr state (Figs 3 and 4, panel B). To check whether this outcome was due to formation of disulfide bridges, we incubated the XccBphP-L193C-F475C protein extracts in reducing conditions, using dithiothreitol (DTT), obtaining the same Pr-locked species (Figure S4). We then sought to find a point substitution in the L193 position as this presents a natural nonrandom variability among distant orthologs (Table S1). It is then possible that different side chains in homologous positions of L193 are fine-tuning the photochemistry in other phytochromes, as evidenced in other reports (27), and that their conservation might be a proxy for relevance. Based on these assumptions, we selected the residues to replace L193 taking into account their frequency and side-chain group type (Table S1). Analyzing the mutational results corresponding to the L193 position, four out of six selected substitutions presented a Pr enrichment (Table 2), indicating that this Leu residue is likely stabilizing the Pfr in XccBphP and that it cannot be easily replaced by any other residues and still retain the Pfr-stabilizing effect. Thus, it is possible that the L193C change in XccBphP-L193C-F475C is sufficient for locking it in Pr; however, this was not further investigated in this work and remains to be confirmed.

A 2-h dark conversion experiment was enough to observe the stabilization behavior toward a photostate, where most variants did not appear to have reached equilibrium (Fig. 4B). A particular case was XccBphP-G454E, which seems to have almost reached equilibrium by the end of the experiment, highly enriched in Pfr. All Pfr-stabilizing variants found, including XccBphP-G454E, are not locked, as the far-red irradiation generates the photoconversion back to the Pr state (Fig. 3B). We then sought to evaluate whether other substitutions in the position 454 would achieve greater Pfr stabilizations or even Pfr-locking, by constructing a random mutagenesis library (G454X).

### Library G454X

The library consists of a mixture of pET24a-XccBphP recombinant plasmids degenerated at the codon corresponding to the 454 position. To generate protein-producing clones, E. coli BL21 (DE3)pLysS were transformed with 100, 10 and 1, ng of DNA, yielding numerous colonies in three cases. To maximize the chance of transforming cells with a single recombinant plasmid, plates derived from the lowest amount of DNA (1 ng) were chosen to continue the screening. 192 colonies were transferred to two 96-well microplates, grown in LB-10% glycerol liquid media at 37°C with agitation (220 rpm) for 16 h and stored at −80°C. 96 colonies (48 from each plate) were selected for the further characterization. The culture growth, the recombinant XccBphP-G454X induction, the clarified crude extract preparation, the BV addition and the 2-h UV-Vis measurements were performed as detailed for the previous experiment in Fig. 2 and panel A from Figs 3–4. Then, 48 of these clones, which showed good spectral quality, were sequenced. 18 substitutions, including the wild-type version, out of the 20 expected substitutions, were represented in the sequenced clones; only G454M and G454K were missing. All of the variants exhibited similar bathy-like photochemical behavior. They were all unlocked, photoconverting upon red and far-red light (Table S3).

In order to deepen the photochemical characterization of these variants, we performed an experiment in which their corresponding clarified crude extract samples were supplemented with BV and illuminated with far-red light (733 nm) during 7 min (taking into account they all present bathy-like behavior). Then, the dark conversion was monitored by acquiring the UV-Vis spectra for ~16 h (Fig. 5). The UV-Vis datasets were then further processed (Fig. 6) and the temporal evolution of Pr and Pfr was fitted to a biexponential function (Fig. 7). Three of the variants (G454R, G454H and G454D) seem to have almost complete BV assembly by the time the dark conversion begins, as evidenced by an increase in the Pr form after the initial time (Fig. 5). For the variants presenting complete BV assemblies, kinetic rates and equilibrium abundances were calculated (Table 3). The kinetic rate was estimated as the time at which the absolute difference between the equilibrium and initial abundances reached half its value, similar to the half-life parameter derived from a monoexponential function and therefore named half-life'.

In these clarified crude extract experiments, the wild-type version presented a similar behavior to the one already reported (17), with a Pfr proportion at the equilibrium value of ~78% and a half-life' of ~309 min (Table 3). Interestingly, only the G454I variant presented a similar behavior to the wild-type. The rest of the characterized substitutions presented a Pfr abundance value at the equilibrium greater than 90% with half-lives’ ranging from 24 to 300 min. There were no substantial differences between the wild-type and the mutants in the wavelengths corresponding to the Pr or Pfr maxima (λmax, Table 3). In order to rank the mutant versions according to their propensity toward the Pfr state, a score was calculated dividing the Pfr abundance at
Figure 4. Screening results: Dark conversion after red light illumination of \textit{Xcc} BphP variants. (A) Scheme of the temporal sequence of light treatment and measurements. The highlighted measurements correspond to the ones used for the qualitative analysis of the dark conversion, shown in panel B. (B) UV-Vis spectra form clarified extracts. The blue, green and red traces correspond to the spectra collected 0 (#2), 60 (#3) and 120 min (#4) incubation in the dark after the red light treatment, respectively. The minimum values were subtracted, and then, each spectrum was normalized to the maximum value in the dataset in the 540-850-nm range (Q bands).
equilibrium by the half-life' (Table 3), where G454E presents the highest score, with a Pfr abundance value at equilibrium of ~99% and a half-life' value of ~24 min.

Validation of the screening approach using the wild-type XccBphP purified protein

As already mentioned, the results of XccBphP Pfr proportion at the equilibrium (~78%) and half-life' value (~308 min) obtained in clarified crude extract experiments were very similar to those already reported for the purified holoprotein (~85% and ~447 min, respectively). The small difference observed may reside, at least, in part, in the data analysis and the experimental conditions such as temperature and the buffer composition. In order to make a better comparison and validate the screening method, we purified wild-type holo-XccBphP, performed the UV-Vis measurements in the same experimental conditions as in clarified crude extracts experiments and subjected the data to
the same data analysis treatments (Fig. 8 and Table 3). The values obtained for pure protein were ~72% and ~315 min, for the Pfr proportion at equilibrium and the half-life, respectively. These results are even in closer agreement to the ones from the screening method using XccBphP clarified crude extract, demonstrating that this strategy is very suitable not only for qualitative, but also for quantitative characterization of phytochrome variants.

**DISCUSSION**

In this article, we present a simple and economic strategy for the simultaneous and rapid characterization of a large number of bacteriophytochrome variants from overexpressing *E. coli* extracts. This method provided high-quality absorption spectra suitable not only for qualitative, but also for quantitative studies. The strategy is based on the fact that rich information of the photochemical behavior of the photoactive proteins resides in the visible range, where bacterial extracts do not significantly absorb. This approach may be used in other systems as well, such as other phytochrome or other photoreceptor families. Furthermore, this approach was validated with purified protein obtaining very similar results and may be easily adapted to be used in other systems.

Performing a rapid 2-h experiment, the methodology provided qualitative information about three important aspects of the photochemical behavior from different XccBphP variants. First, it allowed us to study the holoprotein assembly, determining that all the variants studied assemble, like the wild-type version, into the Pr form. Second, we could gather information about the direction of the dark conversion, allowing the classification of variants into the Pr- or Pfr-favored categories, which were both represented among the generated variants. Finally, we were able to cluster them into slow or fast categories, according to their dark conversion rates. This characterization allowed us to identify the key position 454 located in the tongue of the PHY domain, which dramatically stabilizes the Pfr form.

Subsequently, in a 16-h experiment, quantitative information about the dark conversion process could be gathered on multiple variants simultaneously. This Pr/Pfr abundance plot along time could be modeled with an exponential equation, where the curve’s plateau represents the composition at the equilibrium and the curvature or half-life describes the dark conversion rate. This approach was applied to the mutant random
library G454X, obtaining 18 out of 20 possible substitutions and where all of them produced bathy-like phytochromes. Interestingly, all G454 variants present a higher Pfr proportion at equilibrium or a higher dark conversion rate than the wild-type. The fact that a wide range of residues of varied chemical nature in G454 substitutions favors the Pfr form could be indicative of a destabilization of the Pr form rather than a stabilization of the Pfr form. There is probably a tight constraint for flexibility in the Pr state at the G454 position, which is located in a loop belonging to the tongue region. However, to gain insight into the role of the G454 position, further biophysical studies need to be performed, which are beyond the scope of this work. To finish, the method presented here allowed us to identify several XccBphP variants with extremely favored Pfr and, therefore, good candidates for future structural studies.

Figure 7. Library G454X: Kinetics of the Pr-to-Pfr dark conversion from G454X variants. Using the processed data from Fig. 3, Pr and Pfr individual abundance (%) at each time was calculated for all variants, assuming that these two species are responsible for the absorbance changes during the experiment. Pr/Pfr abundance values were fitted to a double exponential function (eq. 1, black traces). Half-life values and Pr/Pfr abundances at equilibrium were estimated using the fitted coefficients and equation 1.
Table 3. Kinetic analysis for wild-type and G454X variants in dark conversion after far-red irradiation experiments. All entries derive from Fig. 7 (clarified protein extracts) calculations except WT-purified (purified holoprotein) that derives from Fig. 8 calculations.

| Variant    | Half-life (min) | Pr eq. (%) | Pfr eq. (%) | Pr \(\lambda_{\text{max}}\) (nm) | Pfr \(\lambda_{\text{max}}\) (nm) | Pfr eq. (%)/Half-life' (min) |
|------------|----------------|------------|-------------|----------------------------------|----------------------------------|------------------------------|
| WT-purified| 314.8          | 27.6       | 72.4        | 683.9                            | 756.7                            | 0.23                         |
| WT         | 309.4          | 21.7       | 78.3        | 682.0                            | 755.0                            | 0.25                         |
| G454E      | 24.1           | 0.8        | 99.2        | 687.4                            | 749.8                            | 0.12                         |
| G454A      | 32.4           | 2          | 98          | 686.9                            | 749.9                            | 3.02                         |
| G454Q      | 41.9           | 0.9        | 99.1        | 687.7                            | 750.0                            | 2.37                         |
| G454N      | 43.4           | 1.4        | 98.6        | 687.0                            | 749.9                            | 2.27                         |
| G454S      | 48.9           | 0          | 100         | 687.2                            | 750.2                            | 2.04                         |
| G454W      | 61.6           | 3.2        | 96.8        | 686.9                            | 753.1                            | 1.57                         |
| G454C      | 64.8           | 4.3        | 95.7        | 687.1                            | 751.4                            | 1.48                         |
| G454T      | 70.7           | 3.1        | 96.9        | 688.0                            | 749.5                            | 1.37                         |
| G454V      | 71.3           | 2          | 98          | 687.0                            | 753.4                            | 1.37                         |
| G454L      | 73.8           | 0.8        | 99.2        | 689.1                            | 748.9                            | 1.34                         |
| G454F      | 113.1          | 6.3        | 93.7        | 688.6                            | 751.9                            | 0.83                         |
| G454P      | 151.7          | 8.3        | 91.7        | 687.4                            | 751.9                            | 0.60                         |
| G454Y      | 171.8          | 8.4        | 91.6        | 687.7                            | 753.4                            | 0.53                         |
| G454H      | 297.3          | 18.1       | 81.9        | 682.2                            | 757.1                            | 0.28                         |

Figure 8. Validation of the screening approach using the wild-type Xcc BphP purified protein: Dark conversion after far-red illumination of Xcc BphP-WT. A purified holoprotein protein sample (1 mg per ml) was placed in a quartz cuvette, irradiated for 20 min with far-red light and then incubated in the dark. Left panel: UV-Vis spectra were recorded for a period of 1380 min, normalized and represented in jet color code. Middle panel: Example at WT. A purified holoprotein protein sample (1 mg per ml) was placed in a quartz cuvette, irradiated for 20 min with far-red light and then incubated in the dark for the Promotion of Science and Technology of Argentina (ANPCyT), the Argentinian Research Council (CONICET), and the National Agency for the Promotion of Science and Technology of Argentina (ANPCyT), under the grant PICT 2016-1425.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. Degenerate codon number 454 from the Xcc BphP-G454X library.
Figure S2. Xcc BphP variants overexpression.
Figure S3. Screening conditions optimization.
Figure S4. Effect of reducing agent on the Xcc BphP cysteine variant L193C-F475C. Clarified Xcc BphP L193C-F475C extracts were incubated with BV and different concentrations of DTT for 20 min.
Figure S5. Spectral analysis from G454X variants multiwell samples.

Table S1. Frequency analysis of amino acid conservation from a diverse set of 748 orthologs at the corresponding homologous positions to Xcc BphP-L193.
Table S2. Oligonucleotides used in this study.
Table S3. G454X library screening.

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