



**Photoproduct of DXCF cyanobacteriochromes without reversible Cys ligation is destabilized by rotating ring twist of the chromophore**

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1 **Title**

2 **Photoproduct of DXCF cyanobacteriochromes without reversible Cys ligation is**  
3 **destabilized by rotating ring twist of the chromophore**

4

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**17 Abstract**

18 Cyanobacteriochrome photoreceptors (CBCRs) ligate linear tetrapyrrole chromophore via its  
19 first (canonical) Cys residue and show reversible photoconversion triggered by light-  
20 dependent *Z/E* isomerization of the chromophore. Among the huge repertoire of the CBCRs,  
21 DXCF CBCRs contain a second Cys residue within the highly conserved Asp-Xaa-Cys-Phe  
22 (DXCF) motif. In the typical receptors, the second Cys covalently attaches to the 15*Z*-  
23 chromophore in the dark state and detaches from the 15*E*-chromophore in the photoproduct  
24 state, whereas atypical ones that lack reversible ligation activity show red-shifted absorption  
25 in the dark state due to more extended  $\pi$ -conjugated system. Moreover, some DXCF CBCRs  
26 show blue-shifted absorption in the photoproduct state due to the twisted geometry of the  
27 rotating ring. During the process of rational color tuning of a certain DXCF CBCR, we  
28 unexpectedly found that twisted photoproducts of some variant molecules showed dark  
29 reversion to the dark state, which prompted us to hypothesize that the photoproduct is  
30 destabilized by twisted geometry of the rotating ring. In this study, we comprehensively  
31 examined the photoproduct stability of twisted and relaxed molecules derived from the same  
32 CBCR scaffolds under the dark condition. In the DXCF CBCRs lacking reversible ligation  
33 activity, the twisted photoproducts showed faster dark reversion than the relaxed ones,  
34 supporting our hypothesis. By contrast, in the DXCF CBCRs with reversible ligation activity,  
35 the twisted photoproducts showed no detectable photoconversion. Reversible Cys adduct  
36 formation thus results in drastic rearrangement of the protein–chromophore interaction in the  
37 photoproduct state, which would contribute to the previously unknown photoproduct stability.

## 39 Introduction

40 Cyanobacteriochrome photoreceptors (CBCRs), found only in cyanobacteria, belong to a  
41 photoreceptor superfamily that includes linear tetrapyrrole chromophores (or bilin pigments),  
42 such as phycocyanobilin (PCB) and phycoviolobilin (PVB). The range of light wavelength  
43 absorbed by the CBCRs is determined by the effective length of the  $\pi$ -conjugated system in  
44 the chromophores. This length depends on binding chromophore species and protein–  
45 chromophore interaction.<sup>1–25</sup> CBCRs are covalently bound to C3<sup>1</sup> position of the  
46 chromophore via highly conserved “first” (or “canonical”) Cys residue<sup>26–29</sup> similar to the  
47 related plant-type phytochromes (Figs. 1A–D and S1A, B).<sup>30–32</sup> They show reversible  
48 photoconversion triggered by light-dependent *Z/E* isomerization of a double bond between  
49 C15–C16 in the chromophore (Figs. 1A–D and S1A, B). A huge repertoire of CBCRs  
50 showing photoreaction in a wide range of wavelength has been identified<sup>33,34</sup> and  
51 characterized in detail.<sup>26–29,35</sup>

52 CBCRs are categorized into many subfamilies, with many of them applying “second”  
53 (or “DXCF”) Cys residue in the highly conserved Asp-Xaa-Cys-Phe (DXCF) motif as a  
54 crucial color tuner possessing a dual role, i.e., isomerization activity from PCB to PVB and  
55 reversible ligation activity to the chromophore. Typical molecules such as TePixJg retain the  
56 dual role and show blue/green photocycle (Fig. 1A, B, E and S1A, B).<sup>11,26,28,36</sup> They initially  
57 incorporate PCB as a precursor and then isomerize it to PVB.<sup>37</sup> Moreover, the second Cys  
58 covalently attaches to C10 position in the dark state (or ground state) and detaches in the  
59 photoproduct state (or excited state), reversibly altering effective length of the  $\pi$ -conjugated  
60 system between C-to-D (Fig. 1A, E and S1A) and B-to-D (Fig. 1B, E, and S1B) rings that  
61 absorb blue and green light, respectively. Besides, atypical DXCF CBCRs lacking one of the  
62 two roles have been reported; CBCRs lacking the isomerization activity absorb red-shifted  
63 yellow-to-orange light in the photoproduct state by PCB incorporation (Fig. S2A-i)<sup>7,12,18,21</sup>,

64 whereas the other ones lacking the ligation activity absorb green-to-yellow light in the dark  
65 state without  $\pi$ -conjugation cleavage at C10 position (Fig. 1C, E, and S2A-ii).<sup>3,7,18,24,38</sup> The  
66 first Cys covalently attaches to the C3<sup>1</sup> position in any case (Figs. 1A–D and S1A, B). The  
67 lack of the second Cys ligation activity is caused by replacement of Tyr residue with His next  
68 to the first Cys (Fig. S2A-iii).<sup>38</sup>

69 In addition, some DXCF CBCRs adopt a unique color tuning mechanism, “trapped-  
70 twist” in which twisted geometry of ring D results in blue-shifted teal light absorption in the  
71 photoproduct state in comparison with those of the typical ones (Fig. 1D, E and Fig. S2A-  
72 iv)<sup>3,7,18,24,38</sup> This mechanism has been well supported by theoretical and experimental  
73 evidences based on the chromophore chemistry.<sup>19</sup> The twisted ring D is held by specific Phe  
74 residue(s) on a  $\beta$ -sheet with or without an  $\alpha$ -helix, and replacement of these residues results  
75 in cancellation of the twisted geometry (Figs. 1D, F, and S2A-iv, B).<sup>38,39</sup> We have further  
76 reported that not only the twisted photoproduct state but also the twisted dark state contribute  
77 to additional color tuning, for which two residues near the ring D (Tyr/Leu and Thr/Asn  
78 positions) are crucial (Fig. S2A-ii).<sup>38</sup>

79 Taken together, the spectral diversity of the DXCF CBCRs can be explained by  
80 various combinations of four mechanisms: PCB-to-PVB isomerization activity, second Cys  
81 ligation activity, and regulation of the ring D geometry in the dark state and the photoproduct  
82 state (Fig. S2A).<sup>38</sup>

83 Recently, we have identified a unique molecule, AM1\_1499g1, which belongs to the  
84 DXCF subfamily but has lost the second Cys (Fig. S2B).<sup>38</sup> By comparing it with related  
85 molecules retaining the second Cys, we have successfully identified residues crucial for each  
86 color tuning mechanism and created seven color-tuned variants with a broad wavelength  
87 range derived from a single CBCR scaffold.<sup>38</sup>

88           During the engineering process, we unexpectedly found that the “trapped-twist”  
89 photoproduct states of some variant molecules showed dark reversion to the dark state. Dark  
90 reversion kinetics presumably is associated with the stability of ring D of 15*E*-chromophore  
91 in the photoproduct state. Therefore, it is likely that the twisted ring D is less  
92 thermodynamically stable than the relaxed one. To date, only rare DXCF CBCRs with unique  
93 sequence arrangement have been reported to show dark reversion.<sup>5,7</sup>

94           In this study, to gain insights into general characteristics of dark reversion of the  
95 DXCF CBCRs, we compared dark reversion kinetics of the relaxed and twisted photoproduct  
96 states derived from the same CBCR scaffolds. We found that the twisted photoproduct states  
97 showed faster dark reversion than the relaxed ones, except for the molecules showing  
98 reversible Cys adduct formation, in which both twisted and relaxed photoproduct states were  
99 stable under the dark condition. Possible mechanisms underlying these phenomena are  
100 discussed in the context of molecular structure.

## 102 **Experimental**

### 103 **Bacterial strains and growth media**

104 The *Escherichia coli* strain Mach1 T1<sup>R</sup> (Thermo Fisher Scientific) was used during cloning  
105 of plasmids. The *E. coli* strain C41 (Cosmo Bio) was used for protein expression through the  
106 pKT271 construct as a PCB synthetic system.<sup>40,41</sup> Bacterial cells were grown on Lysogeny  
107 Broth (LB) agar medium containing 20 µg mL<sup>-1</sup> kanamycin with or without 20 µg mL<sup>-1</sup>  
108 chloramphenicol at 37°C. For protein expression, after the optical density at 600 nm of the  
109 cells reached 0.4–0.8, isopropyl β-D-1-thiogalactopyranoside was added (final concentration,  
110 0.1 mM), and the cells were cultured at 18°C overnight before being harvested.

111

### 112 **Bioinformatic analysis.**

113 Multiple sequence alignment and neighbor-joining phylogenetic trees were constructed using  
114 MEGA7 software.<sup>42</sup> Structural information (<sup>15</sup>ZPb\_TePixJg, PDB\_ID: 4GLQ<sup>26</sup>;  
115 <sup>15</sup>EPg\_TePixJg, PDB\_ID: 3VV4<sup>28</sup>; and <sup>15</sup>EPg\_Slr1393g3, PDB\_ID: 5M82<sup>29</sup>) were extracted  
116 from databases and used to compare conformation near the chromophores. Images of  
117 molecular structure were generated by UCSF Chimera software.<sup>43</sup>

118

### 119 **Plasmid construction for protein expression**

120 The plasmids of AM1\_1499g1 (amino acid positions 47–222), AM1\_6305g1 (amino acid  
121 positions 33–203), and AM1\_0048g1 (amino acid positions 199–366) variants were  
122 constructed from their corresponding parent plasmids, which were constituted by insertion of  
123 their corresponding gene fragments fused with N-terminal His-tag sequence into the pET28a  
124 vector (Novagen).<sup>7,38</sup> The plasmids of AM1\_1499g1\_S<sub>118</sub>C, S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N, S<sub>118</sub>C/H<sub>147</sub>Y,  
125 F<sub>97</sub>V/S<sub>118</sub>C, F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N, and F<sub>97</sub>V/S<sub>118</sub>C/H<sub>147</sub>Y, and AM1\_6305g1\_L<sub>132</sub>Y/N<sub>140</sub>T  
126 variants have been constructed in the previous study.<sup>38</sup> The plasmids of AM1\_6305g1\_F<sub>78</sub>V

127 and F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T, and AM1\_0048g1\_F<sub>241</sub>V/F<sub>298</sub>L were generated by site-directed  
128 mutagenesis using corresponding parent plasmids. KOD One PCR Master Mix (Toyobo Life  
129 Science) with appropriate nucleotide primer sets (AM1\_6305g1, forward primer 5'-  
130 GGATGCgtcGTTGCTGAAAAAGTAGTTCCG-3' and reverse primer 5'-  
131 AGCAACgacGCATCCCGTTGTATAGTT-3' for replacement of Phe78 by Val;  
132 AM1\_0048g1, forward primer 5'-GGTACCgtcGTCGCCGAATCTGTTGCCCC-3' and  
133 reverse primer 5'-GGCGACgacGGTACCCTCACCGTTATTGTTGAGG3' for replacement  
134 of Phe241 by Val, and forward primer 5'-GAGCGTctcGCCGTCAAAGCAAACATT-3' and  
135 reverse primer 5'-GACGGCgagACGCTCTAGCAAACCGAT-3' for replacement of Phe298  
136 by Leu; each mutation site is shown by small letters) was used for the mutagenesis. Plasmid  
137 sequences were confirmed by DNA sequencing (Eurofins Genomics). The plasmid of  
138 NpR5113g3 (amino acid positions 388–557) has been constituted in the previous study.<sup>38</sup>

139

#### 140 **Protein extraction and purification**

141 All proteins were expressed in the *E. coli* strain C41 containing pKT271 in 1 L LB. After  
142 expression was induced, the culture broth was centrifuged at 5,000 g for 15 min to collect  
143 cells. The cells were resuspended in the lysis buffer (20 mM HEPES–NaOH pH 7.5, 0.1 M  
144 NaCl and 10% (w/v) glycerol) with 0.5 mM tris(2-carboxyethyl)phosphine. Protein was  
145 purified as described in the previous studies.<sup>7,38</sup> Purified proteins were dialyzed against the  
146 lysis buffer containing 1 mM dithiothreitol (DTT). Protein concentration was determined by  
147 the Bradford method.

148

#### 149 **Electrophoresis and fluorescence detection**

150 Purified proteins were diluted in a buffer (60 mM Tris–HCl pH 8.0, 2% (w/v) sodium  
151 dodecyl sulfate (SDS) and 60 mM DTT), denatured at 95°C for 3 min, and electrophoresed at



152 room temperature (r.t.) using 12% (w/v) SDS polyacrylamide gels. The electrophoresed gels  
153 were soaked in distilled water for 30 min before fluorescence bands were visualized as  
154 previously described.<sup>6,27</sup> Gels were then stained with Coomassie Brilliant Blue R-250.

155

### 156 **Spectroscopy and dark reversion kinetics**

157 Ultraviolet and visible absorption spectra of the proteins were recorded with a UV-2600  
158 spectrophotometer (SHIMADZU) at r.t. or 30°C using a thermostated cuvette holder. An  
159 Opto-Spectrum Generator (Hamamatsu Photonics, Inc.) was used to generate monochromatic  
160 light of various wavelengths to induce photoconversion: Pb form, 390–420 nm; Pt form, 470–  
161 490 nm; Pg form, 490–580 nm; Py form, 580–640 nm.

162 For monitoring dark reversion of CBCRs, the absorbance of photoproduct states and  
163 transition process at each maximum absorption of dark states were chronologically measured  
164 in the dark condition. The half-life was calculated from the dark reversion kinetics.

165

### 166 **Biochemical characterization of cyanobacteriochromes**

167 For denaturation assays, native AM1\_6305g1\_F<sub>78</sub>V and F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T, and  
168 AM1\_0048g1\_F<sub>241</sub>V/F<sub>298</sub>L in both the dark state (15Z-isomer) and photoproduct state (15E-  
169 isomer) were diluted fivefold in 8 M acidic urea (pH < 2.0). Their absorption spectra were  
170 recorded at r.t. before and after 3 min of illumination with white light. Assignment of the  
171 chromophore species was conducted by comparing the spectra between native and each  
172 denatured molecule which incorporates PVB.<sup>7,38</sup>

## 174 **Results**

### 175 **Dark reversion of AM1\_1499g1 variants**

176 In the previous study, we have created the twisted and relaxed molecules derived from the  
177 same AM1\_1499g1 scaffold: AM1\_1499g1\_wild-type (orange/green photocycle),  
178 AM1\_1499g1\_S<sub>118</sub>C (yellow/teal photocycle), S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N (green/teal photocycle) and  
179 S<sub>118</sub>C/H<sub>147</sub>Y (blue/teal photocycle) for the twisted type; AM1\_1499g1\_F<sub>97</sub>V (orange/yellow  
180 photocycle), AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C (yellow/green photocycle), F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N  
181 (green/green photocycle), and F<sub>97</sub>V/S<sub>118</sub>C/H<sub>147</sub>Y (blue/green photocycle) for the relaxed  
182 type.<sup>38</sup> While analyzing spectroscopy of these molecules, we incidentally found that some  
183 variant molecules showed dark reversion, which led us to hypothesize that the twisted ones  
184 are unstable and tend to show dark reversion in comparison with the relaxed ones. To test this  
185 hypothesis, we measured the absorption spectra of the photoproduct states during dark  
186 incubation at 30°C to monitor the dark reversion for 5 hours (Fig. 2 and Table 1). We have  
187 previously reported that the photoproduct state of AM1\_1499g1\_wild-type showed  
188 thermochromism, which reflects an equilibrium between the twisted state and relaxed state.<sup>38</sup>  
189 Therefore, we excluded AM1\_1499g1\_wild-type and its related AM1\_1499g1\_F<sub>97</sub>V from  
190 this analysis.

191 We found that the twisted photoproduct states of AM1\_1499g1\_S<sub>118</sub>C and  
192 AM1\_1499g1\_S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N showed dark reversion under the condition described above  
193 (at 30°C for 5 hours) (Fig. 2A, C), whereas the relaxed counterparts of  
194 AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C and AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N scarcely showed dark  
195 reversion during the observed time range (Fig. 2B, D). We detected an increased absorption  
196 around 580 and 560 nm for AM1\_1499g1\_S<sub>118</sub>C and AM1\_1499g1\_S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N,  
197 respectively (Fig. 2A, C). The dark reversion of AM1\_1499g1\_S<sub>118</sub>C (Fig. 2A) was

198 remarkably faster than that of AM1\_1499g1\_S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N (Fig. 2C). These results  
199 support our hypothesis that the twisted states are more likely to show dark reversion.

200 By contrast, both twisted and relaxed photoproduct states of  
201 AM1\_1499g1\_S<sub>118</sub>C/H<sub>128</sub>Y and F<sub>97</sub>V/S<sub>118</sub>C/H<sub>128</sub>Y showed no detectable dark reversion  
202 during the observed time range. These results suggest a difference in stability of photoproduct  
203 states between the DXCF CBCRs with and without second Cys ligation activity, although the  
204 second Cys ligation occurs in the dark state instead of the photoproduct state. Although the  
205 absorbance around 415 nm of both molecules slightly increased with time, we could not  
206 detect clear isosbestic points around 360 nm (Fig. 2E, F). Therefore, this slight increase of  
207 absorbance was not due to the dark reversion but rather due to the light scattering caused by  
208 protein aggregation under relatively high temperature of 30°C.

209

### 210 **Dark stability of the photoproduct states of the molecules lacking ligation activity of** 211 **second Cys**

212 To further investigate the relationship between the ring D geometry and the dark reversion  
213 kinetics, we focused on green/teal-reversible AM1\_6305g1, a close homolog of  
214 AM1\_1499g1, which lacks the ligation activity of the second Cys and forms a subfamily with  
215 blue/teal reversible (NpR1597g1<sup>18</sup> and NpR5113g1<sup>18</sup>) and green/teal reversible (FdDpxAg<sup>24</sup>)  
216 CBCRs (Fig. S2B). In the previous study, we have constructed a variant molecule,  
217 AM1\_6305g1\_L<sub>132</sub>Y/N<sub>140</sub>T, which showed yellow/teal reversible photoconversion<sup>38</sup> The  
218 teal-absorbing photoproduct states of these two molecules appeared to be the twisted type.  
219 Thus, we introduced F<sub>78</sub>V replacement in these two molecules to cancel the trapped geometry  
220 of ring D, generating the relaxed type of variants, AM1\_6305g1\_F<sub>78</sub>V and  
221 AM1\_6305g1\_F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T (Fig. S2C–E). Consequently, the photoproduct states of  
222 these two molecules are about 20–30 nm red shifted in absorbing green light in comparison

223 with those of the parent molecules (Fig. S2D, E),<sup>7,38</sup> confirming that these photoproduct states  
224 adopt the relaxed ring D geometry.

225 We next examined the dark stability of the photoproduct states of these four  
226 molecules under the same condition as the experiments for AM1\_1499g1 variants (i.e.  
227 monitoring for 5 hours at 30°C) (Fig. 3A–D). As expected, the twisted photoproduct states of  
228 the wild-type and AM1\_6305g1\_L<sub>132</sub>Y/N<sub>140</sub>T showed dark reversion, whereas the relaxed  
229 states of AM1\_6305g1\_F<sub>78</sub>V and AM1\_6305g1\_F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T scarcely showed dark  
230 reversion (Fig. 3A–D and Table 1). This observation is notably consistent with that from  
231 AM1\_1499g1, supporting our hypothesis that the twisted ring D is less thermodynamically  
232 stable than the relaxed one.

233

#### 234 **Dark reversion kinetics**

235 Dark reversion half-life of the variant molecules was calculated from the slope of absorbance  
236 increase during each transition process in the logarithmic plots to quantitatively estimate the  
237 dark reversion kinetics of the variant proteins showing the detectable dark reversion (Fig. 4  
238 and Table 1). Half-life for AM1\_1499g1\_S<sub>118</sub>C and S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N (twisted type) was  
239 5.99 h ± 0.31 and 22.6 h ± 1.31, respectively, whereas that for F<sub>97</sub>V/S<sub>118</sub>C and  
240 F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N (relaxed type) was 52.6 h ± 3.05 and 53.1 h ± 35.8, respectively (Fig.  
241 4A, B and Table 1). Moreover, the half-life for AM1\_6305g1 and L<sub>132</sub>Y/N<sub>140</sub>T (twisted type)  
242 was 31.8 h ± 4.59 and 131.0 h ± 24.6, respectively, whereas that for F<sub>78</sub>V and  
243 F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T (relaxed type) was 85.5 h ± 11.5 and 262.1 h ± 85.7, respectively (Fig. 4C,  
244 D and Table 1). Statistical analyses showed that the kinetics of the DXCF CBCRs  
245 incorporating the “twisted” chromophore were significantly more rapid than that of the  
246 DXCF CBCRs incorporating the “relaxed” one (Fig. 4 and Table 1).

247

248 **Dark stability of the photoproduct states of the molecules with reversible Cys adduct**  
249 **formation irrespective of ring D configuration**

250 We found that neither AM1\_1499g1\_S<sub>118</sub>C/H<sub>128</sub>Y nor F<sub>97</sub>V/S<sub>118</sub>C/H<sub>128</sub>Y showed detectable  
251 dark reversion during the observed time range (Fig. 2E, F), suggesting that second Cys-  
252 adducting capability contributes to the photoproduct stability, whether the photoproduct ring  
253 D is twisted or relaxed. To test this hypothesis, we focused on NpR5113g3, which forms a  
254 subfamily with AM1\_1499g1 and AM1\_6305g1 (Fig. S2B) but showed reversible Cys  
255 adduct formation with blue/teal photocycle (Fig. S3).<sup>18</sup> As expected, similar to  
256 AM1\_1499g1\_S<sub>118</sub>C/H<sub>128</sub>Y, the twisted photoproduct state of NpR5113g3 did not show  
257 detectable dark reversion (Fig. 2E and S3).

258 To test our hypothesis on molecules outside of this subfamily, we focused on another  
259 CBCR, AM1\_0048g1. AM1\_0048g1 belongs to a subfamily distinct from the AM1\_1499g1  
260 subfamily (Fig. S2B) while retaining the DXCF Cys residue that shows reversible Cys adduct  
261 formation with blue/teal photocycle similar to NpR5113g3. We monitored its dark reversion  
262 process from the teal-absorbing photoproduct state to the blue-absorbing dark state (Fig. 3E).<sup>7</sup>  
263 We found that the twisted photoproduct state of AM1\_0048g1 did not show detectable dark  
264 reversion, similar to AM1\_1499g1\_S<sub>118</sub>C/H<sub>128</sub>Y and NpR5113g3 (Figs. 2E, 3E, and S3 and  
265 Table 1). Moreover, we constructed a variant protein, AM1\_0048g1\_F<sub>241</sub>V/F<sub>298</sub>L, to cancel  
266 the twisted ring D geometry of the photoproduct state. The photoproduct state of  
267 AM1\_0048g1\_F<sub>241</sub>V/F<sub>298</sub>L is 8 nm red shifted in comparison with that of the wild-type  
268 protein (Fig. S2C, F).<sup>7</sup> As expected, the relaxed photoproduct state of this variant did not  
269 show detectable dark reversion (Fig. 3F and Table 1).

## 271 **Discussion**

272 In this study, we investigated the relationship between the photoproduct ring D geometry  
273 (twisted or relaxed) of the PVB chromophore and the dark reversion kinetics of the DXCF  
274 CBCRs. In the DXCF CBCRs without reversible Cys adduct formation, the twisted  
275 photoproduct states, but not the relaxed photoproduct states, showed dark reversion to the  
276 dark states (Figs. 2A–D and 3A–D). These results strongly support our hypothesis that  
277 twisted geometry of the rotating ring D results in instability of the photoproduct state,  
278 promoting dark reversion. By contrast, in the DXCF CBCRs with reversible Cys adduct  
279 formation, neither twisted nor relaxed state showed detectable dark reversion (Figs. 2E, F, 3E,  
280 F, and S3). These results indicate that reversible ligation activity of the second Cys in the  
281 dark state affects the photoproduct stability, although the photoproduct state does not form a  
282 covalent bond with the second Cys. Therefore, positioning of the second Cys residue in the  
283 photoproduct state may vary between the molecules with and without reversible Cys  
284 adducting capability. In fact, it has been reported that the second Cys of TePixJg, a typical  
285 DXCF CBCR with reversible Cys-adduct formation, showed large conformational change  
286 upon photoconversion.<sup>26,28</sup> Particularly, the side chain of the free second Cys in the  
287 photoproduct state faces a direction opposite from the chromophore (Fig. S1A, B). This  
288 movement would result in structural changes of the conserved Asp residue in the DXCF  
289 motif that interacts with the ring D nitrogen. Such dynamic rearrangement of the second Cys  
290 and Asp residues may also occur in the molecules analyzed here, which may contribute to  
291 stabilization of the twisted ring D in the photoproduct state. Structural studies are needed to  
292 address the detailed molecular mechanisms underlying these phenomena.

293 We have previously found that atypical DXCF CBCRs with unique sequence  
294 arrangement showed dark reversion; cce\_4193g1 retains the second Cys but not the first  
295 Cys<sup>5</sup>; AM1\_1870g4 has an arranged DXCF motif, in which the Asp residue is swapped with

296 the next residue.<sup>7</sup> Our previous study suggested that the chemical environment near the  
297 DXCF motif affects the 15E–chromophore stability, consistent with the observation in this  
298 study.

299 It has been reported that some molecules belonging to the extended red/green (XRG)  
300 CBCR subfamily show dark reversion from the green-absorbing photoproduct state to the  
301 red-absorbing dark state, whereas others do not.<sup>6,20,44</sup> Previous structural and spectral studies  
302 elucidated that the photoproduct state of the XRG CBCRs adopted highly twisted ring D,  
303 which absorbs shorter-wavelength green light despite PCB incorporation (Fig. S1C).<sup>29,35</sup> This  
304 twisted ring D is fixed by the two Phe residues on the  $\alpha$ –helix and the  $\beta$ –sheet conserved in  
305 many XRG CBCRs (Fig. S1C and S2B). Replacement of these Phe residues with non-  
306 aromatic ones, such as Val and Leu, results in red shift of the photoproduct absorption  
307 compared with their parent molecules.<sup>39,45</sup> Although dark reversion kinetics of these variant  
308 molecules remains unclear, a trend similar to that observed in this study should be expected.

309 On the other hand, some atypical XRG CBCRs categorized into insert Cys subfamily  
310 have another second Cys residue within an inserted loop structure, which has a reversible  
311 ligation activity as well as the second Cys of the DXCF CBCRs.<sup>17,23,33,46</sup> Cho et al. have  
312 reported that Mbr3854g4 (UG1) and Mbl3738g2 (UG2) of the insert Cys CBCRs did not  
313 show detectable dark reversion.<sup>46</sup> UG1 and UG2 possess aromatic amino acids on the  $\alpha$ –helix  
314 and the  $\beta$ –sheet, whose replacement resulted in red-shift of the photoproduct, indicating that  
315 trapped-twist model is also applicable to this subfamily.<sup>46</sup> In summary, the twisted insert Cys  
316 CBCRs with the reversible Cys ligation activity did not show dark reversion, which is  
317 comparable to the twisted DXCF CBCRs with the reversible Cys ligation activity. Lack of  
318 the ligation activity is again suggested to be the driving force of the photoproduct instability.

319 To date, color tuning by replacing specific Phe residue(s) has been done in the DXCF  
320 CBCRs in the AM1\_1499g1 subfamily and some XRG CBCRs.<sup>38,39,45</sup> The photoproduct

321 states of these variant molecules are red shifted in comparison with those of their parent  
322 molecules due to cancellation of the ring D twist. In this study, we successfully canceled the  
323 trapped-twist of the photoproduct state of two different DXCF CBCR scaffolds,  
324 AM1\_6305g1 and AM1\_0048g1 (Fig. S2C–E). As AM1\_0048g1 belongs to a subfamily  
325 distinct from the AM1\_1499g1 subfamily (Fig. S2B), our results provide a new approach to  
326 modify sensing light quality of a wide variety of CBCRs, which will improve fine tuning of  
327 the optogenetic and fluorescent templates.<sup>6,20,27,38,44,47–49</sup>



329 **Conflicts of interest**

330 There are no conflicts to declare.

331

332 **Acknowledgments**

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493 **Table 1. Photochemical properties of DXCF CBCRs in twisted vs. relaxed states\*.**

	Photocycle		D-ring geometry	Dark state 15Z, $\lambda_{\text{max}}$ (nm)	Photoproduct state 15E, $\lambda_{\text{max}}$ (nm)	Kinetics		t-test P value
	Dark	Photo				DR, Half life (h) <sup>#, Δ</sup>		
AM1_1499g1_S118C	Yellow	Teal	Twisted	577	492	6.0 ± 0.3	} < 0.001	
AM1_1499g1_F97V/S118C	Yellow	Green	Relaxed	577	515	52.6 ± 3.1		
AM1_1499g1_S118C/Y151L/T159N	Green	Teal	Twisted	559	492	22.6 ± 1.3	} < 0.05	
AM1_1499g1_F97V/S118C/Y151L/T159N	Green	Green	Relaxed	560	524	53.1 ± 35.8		
AM1_1499g1_S118C/H147Y	Blue	Teal	Twisted	413	492	n.d.	-	
AM1_1499g1_F97V/S118C/H147Y	Blue	Green	Relaxed	417	513	n.d.		
AM1_6305g1_L132Y/N140T	Yellow	Teal	Twisted	576	491	131.0 ± 24.6	} < 0.05	
AM1_6305g1_F78V/L132Y/N140T	Yellow	Green	Relaxed	576	513	262.1 ± 85.7		
AM1_6305g1	Green	Teal	Twisted	558	492	31.8 ± 4.6	} < 0.005	
AM1_6305g1_F78V	Green	Green	Relaxed	559	523	85.5 ± 11.5		
AM1_0048g1	Blue	Teal	Twisted	417	498	n.d.	-	
AM1_0048g1_F241V/F298L	Blue	Green	Relaxed	421	507	n.d.		

494 The wild-type molecules of these DXCF CBCRs are categorized into the groups 1 or 2 shown  
 495 in Fig. S2B.

496 \*These parameters were measured at 30°C.

497 <sup>#</sup>Half-life of the dark reversion (DR) was calculated from the slope in the logarithmic plots  
 498 shown in Fig. 4. These values are presented as mean ± standard deviation (n = 3 to 6).  
 499 Significant difference between the half-lives of DXCF CBCRs of twisted and relaxed types  
 500 was analyzed by Student's *t*-test.

501 <sup>Δ</sup>n/d means “not determined.”

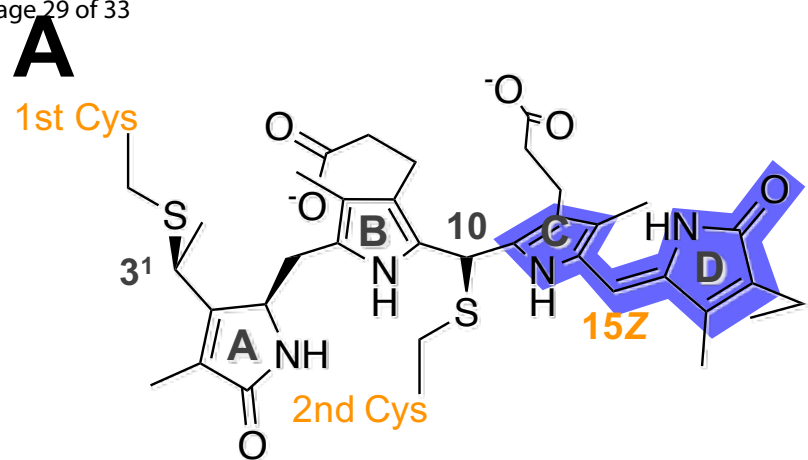
503 **Figures**

504 **Fig. 1. Stereochemistry of phycoviolobilin (PVB).** (A–D) Chemical structures of 15*Z*- and  
505 15*E*-PVB incorporated into DXCF CBCRs. “First” (or “canonical”) Cys covalently binds to  
506 the C3<sup>1</sup> position of the chromophore, whereas “second” (or “DXCF”) Cys reversibly attaches  
507 to/detaches from C10 position with photoconversion/dark reversion induced by *Z/E*  
508 isomerization at the C15 position. The attachment of the second Cys residue to 15*Z*-PVB in  
509 the dark state (or ground state) (A) and the detachment from 15*E*-PVB of the residue in the  
510 photoproduct state (or excited state) (B) are observed in typical DXCF CBCRs. In some  
511 atypical DXCF CBCRs, red-shifted absorption caused by the  $\pi$ -conjugated system extended  
512 to B-to-D rings in the dark state is observed due to lack of the ligation activity (C). Moreover,  
513 some DXCF CBCRs show blue-shifted absorption caused by the  $\pi$ -conjugated system  
514 restricted to B-to-C rings in the photoproduct state due to the twisted geometry of the D-ring,  
515 called “trapped-twist” mechanism (D).  $\pi$ -conjugated systems are highlighted by  
516 corresponding color of their absorption light. (E) Comparison of absorption spectra between  
517 the typical and atypical DXCF CBCRs shown in Fig. 1A–D (upper: blue light-absorbing  
518 15*Z*-dark state (<sup>15*Z*</sup>Pb) and green light-absorbing 15*E*-photoproduct state (<sup>15*E*</sup>Pg); middle:  
519 green light-absorbing 15*Z*-dark state (<sup>15*Z*</sup>Pg); lower: teal light-absorbing 15*E*-photoproduct  
520 state (<sup>15*E*</sup>Pt)). (F) Positions of specific Phe residues (green) on an  $\alpha$ -helix and a  $\beta$ -sheet  
521 (shown in Fig. S2B) of twisted DXCF CBCRs, which contribute to stabilization of a 15*E*-  
522 chromophore (light gray). These positions are shown using structural information of TePixJg,  
523 a typical DXCF CBCR with 15*E*-PVB incorporated (PDB\_ID: 3VV4)<sup>28</sup>. Protein structure is  
524 shown by stick and sphere models with the secondary structure shown by the ribbon model.

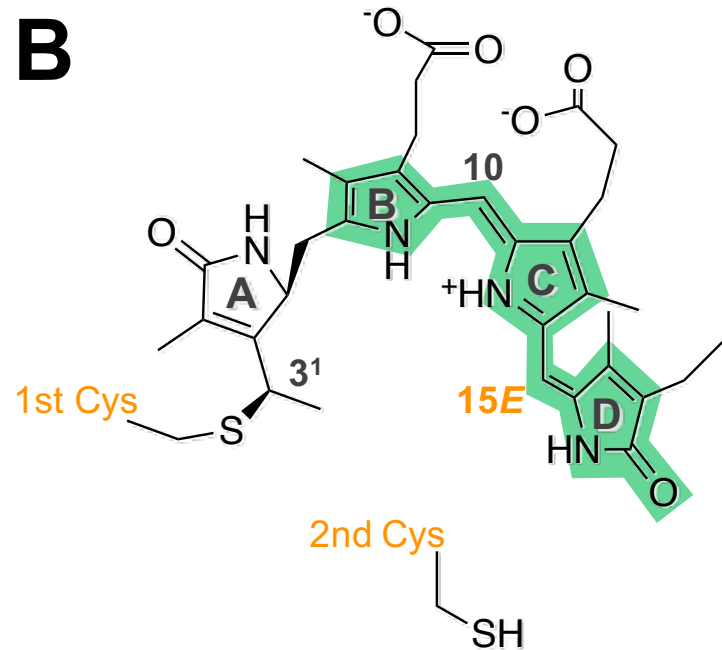
526 **Fig. 2. Reversible photoconversion and dark reversion of AM1\_1499g1 variants.** (A, C,  
527 E) Dark reversion process of (A) AM1\_1499g1\_S<sub>118</sub>C (yellow/teal photocycle), (C)  
528 AM1\_1499g1\_S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N (green/teal photocycle) and (E) AM1\_1499g1\_S<sub>118</sub>C/H<sub>147</sub>Y  
529 (blue/teal photocycle) with twisted ring D of 15*E*-PVB (cyan to dark blue). (B, D, F) Dark  
530 reversion process of (B) AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C (yellow/green photocycle), (D)  
531 AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N (green/green photocycle) and (F)  
532 AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C/H<sub>147</sub>Y (blue/green photocycle) with relaxed ring D of 15*E*-PVB  
533 (magenta to dark red). The absorption spectra were measured at 30°C and normalized against  
534 each maximum absorption of their dark states (light gray). The transition process from the  
535 photoproduct states to the dark states was monitored at 0, 1, 2, 3, 4, and 5 h in dark condition.

537 **Fig. 3. Reversible photoconversion and dark reversion of AM1\_1499g1 homologs.** (A, C,  
538 E) Dark reversion process of (A) AM1\_6305g1 (green/teal photocycle), (C)  
539 AM1\_6305g1\_L<sub>132</sub>Y/N<sub>140</sub>T (yellow/teal photocycle) and (E) AM1\_0048g1 (blue/teal  
540 photocycle) with twisted ring D of 15*E*-PVB (cyan to dark blue). (B, D, F) Dark reversion  
541 process of (B) AM1\_6305g1\_F<sub>78</sub>V (green/green photocycle), (D)  
542 AM1\_6305g1\_F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T (yellow/green photocycle) and (F)  
543 AM1\_0048g1\_F<sub>241</sub>V/F<sub>298</sub>L (blue/green photocycle) with relaxed ring D of 15*E*-PVB  
544 (magenta to dark red). The absorption spectra and transition process were observed under the  
545 same conditions shown in Fig. 2 and normalized against each maximum absorption of their  
546 dark states (light gray).

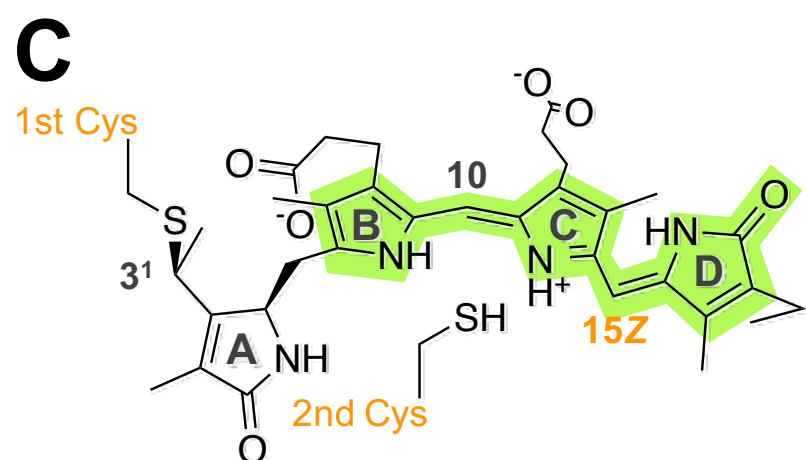
548 **Fig. 4. Dark reversion kinetics of DXCF CBCRs in twisted/relaxed types.** The logarithm  
549 of normalized difference absorbance ( $\Delta A(t)/\Delta A(0)$ ;  $\Delta A(t)$  = transition process – 15Z–dark  
550 state,  $\Delta A(0)$  = 15E–photoproduct state – 15Z–dark state), at each time point (h) was plotted  
551 as mean  $\pm$  standard deviation (n = 3 to 6). DXCF CBCRs in twisted type (cyan) and relaxed  
552 type (magenta), and significant difference between them analyzed by the Student's *t*-test ( $*P$   
553  $< 0.05$ ,  $**P < 0.005$ , and  $***P < 0.001$ , respectively) are shown in each plot. (A)  
554 AM1\_1499g1\_S118C and F<sub>97</sub>V/S<sub>118</sub>C; (B) AM1\_1499g1\_S118C/Y<sub>151</sub>L/T<sub>159</sub>N and  
555 F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N; (C) AM1\_6305g1 and F<sub>78</sub>V; (D) AM1\_6305g1\_L<sub>132</sub>Y/N<sub>140</sub>T and  
556 F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T. Half-life was calculated from the slope of each plot. The absorbance of the  
557 proteins was measured at the wavelength of each maximum absorption of the dark states  
558 (peak area of their absorption spectra shown in Figs. 2 and 3).



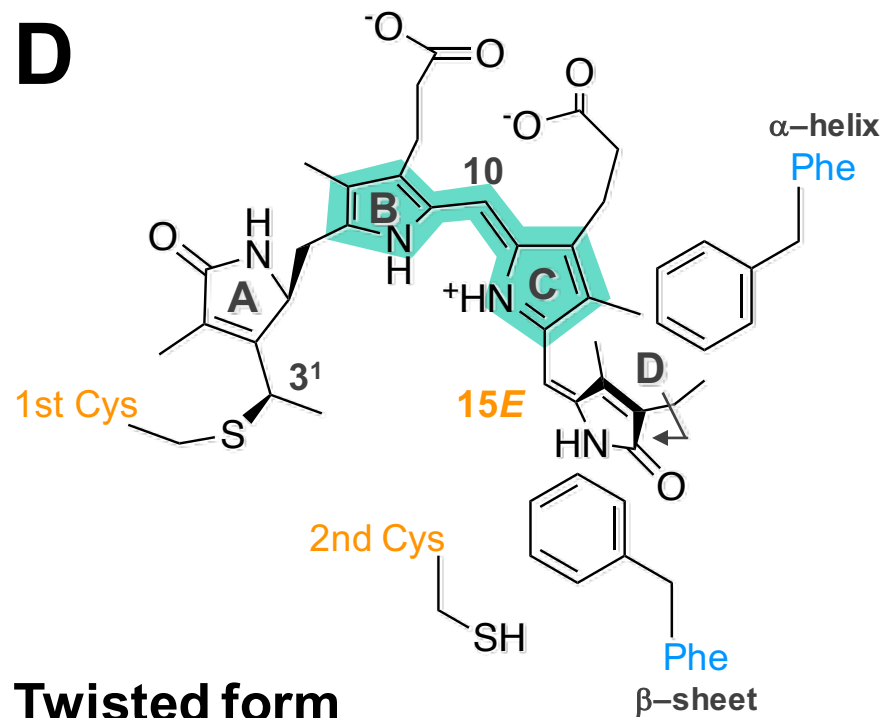
Attached form



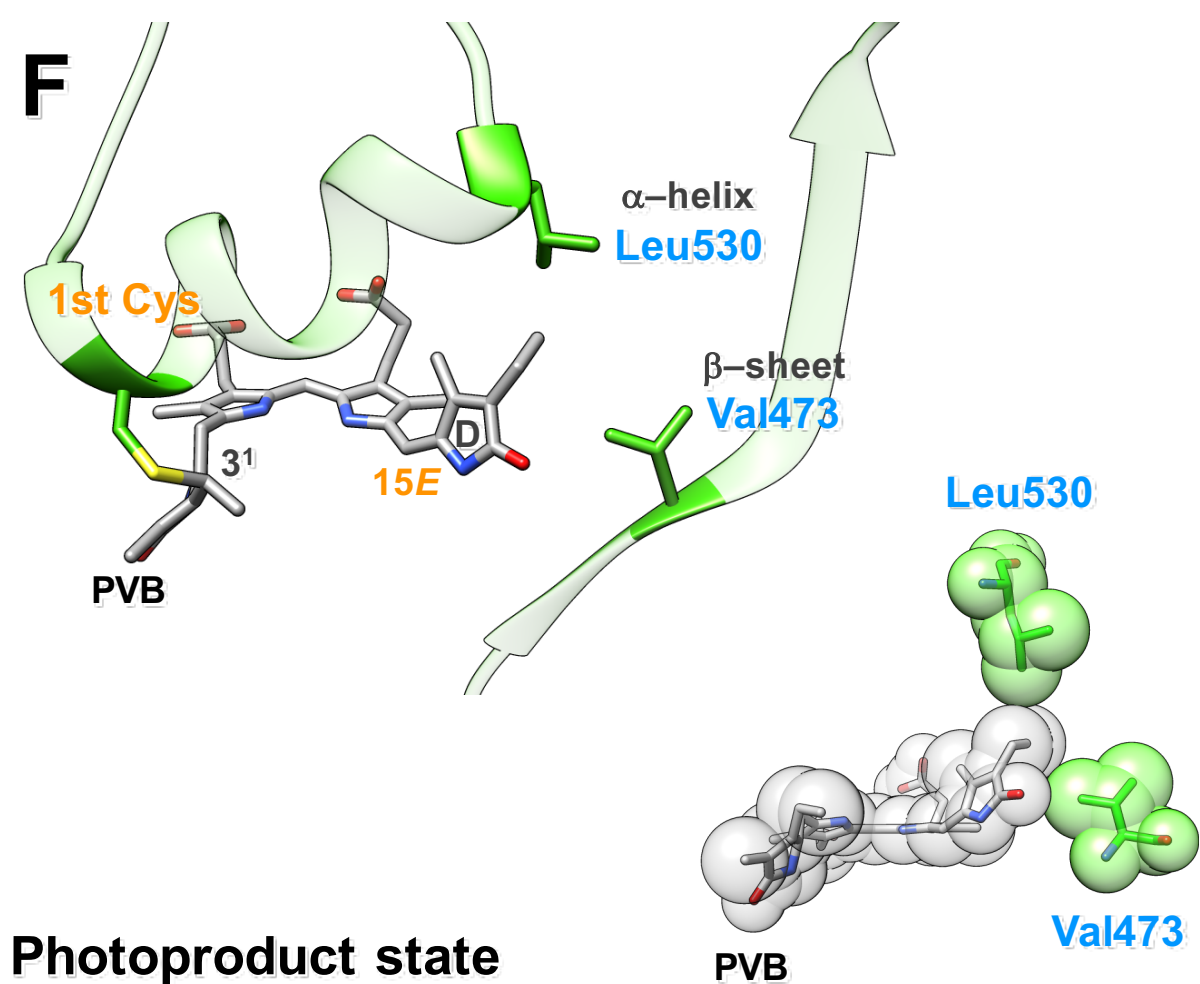
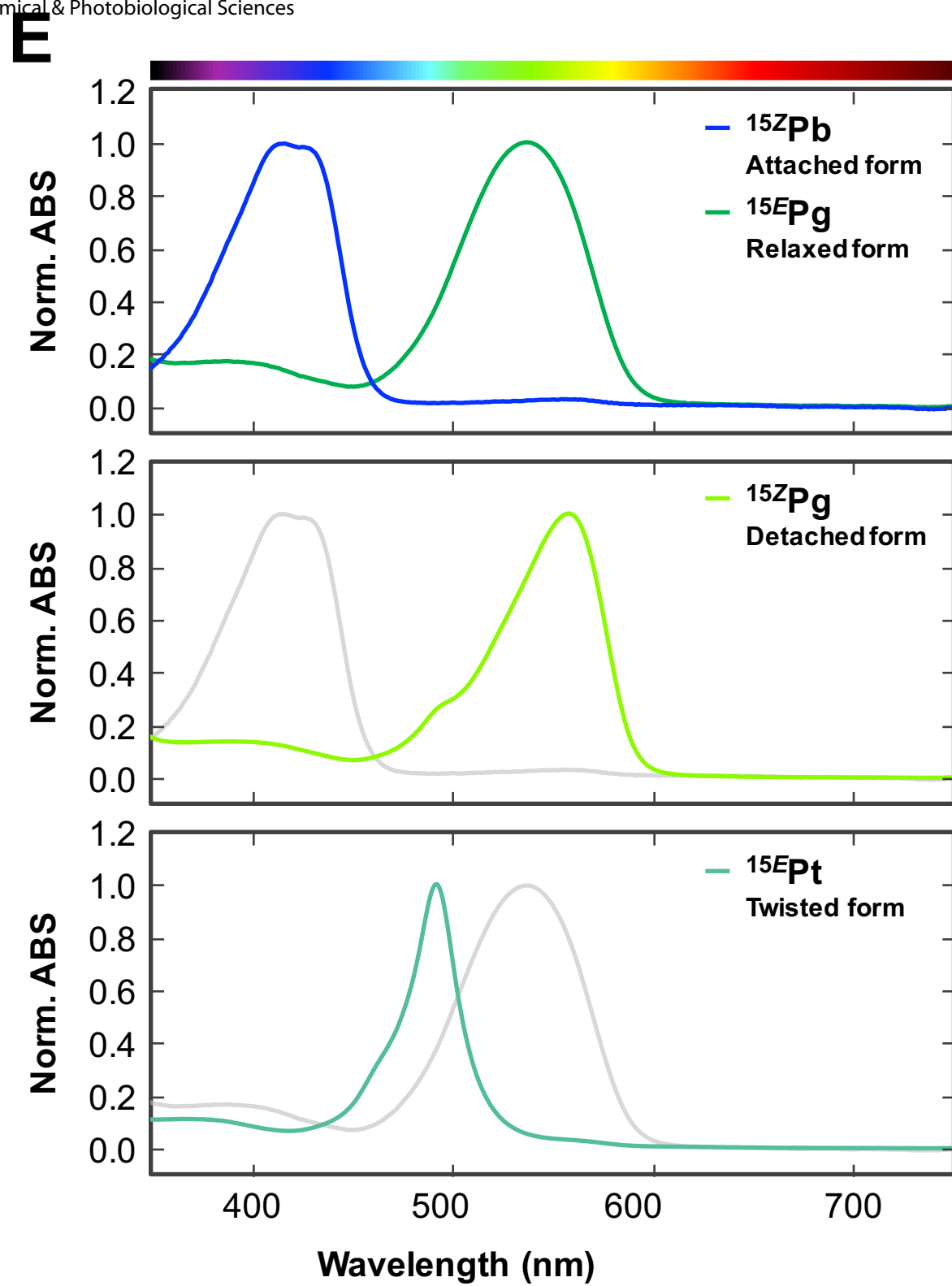
Relaxed form



Detached form



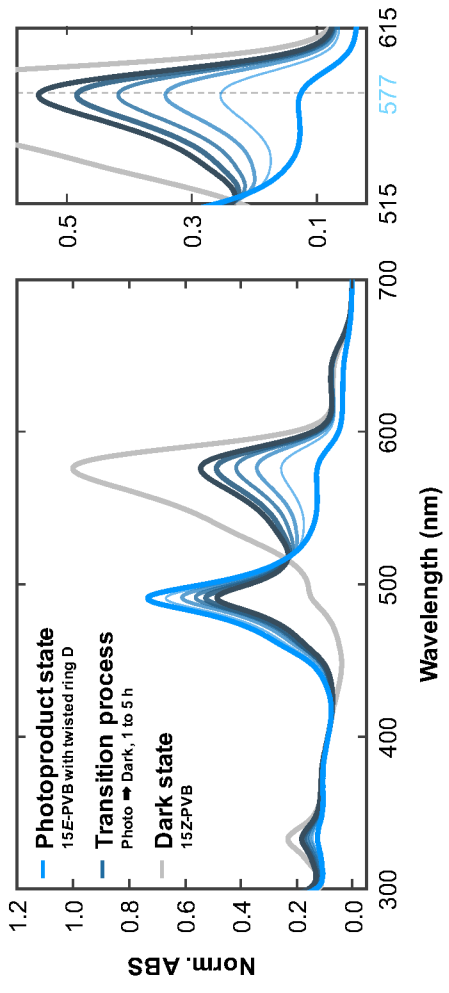
Twisted form



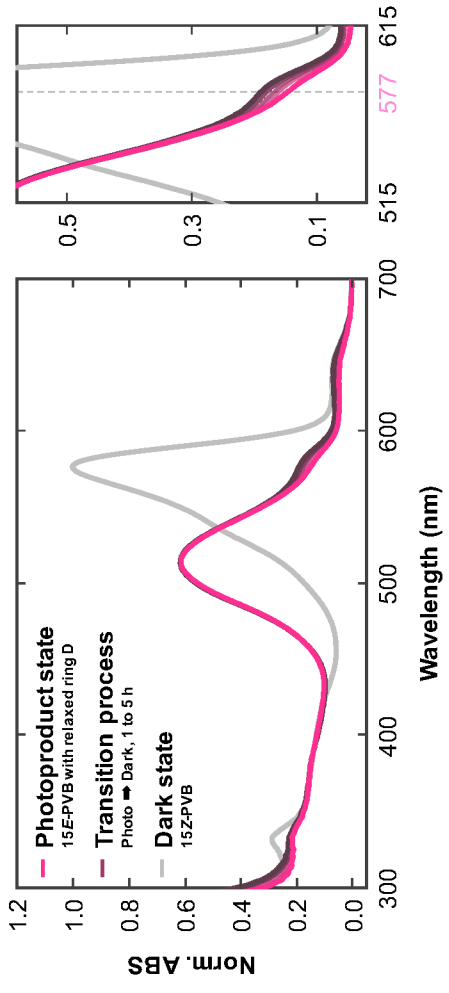
Photoproduct state

PVB

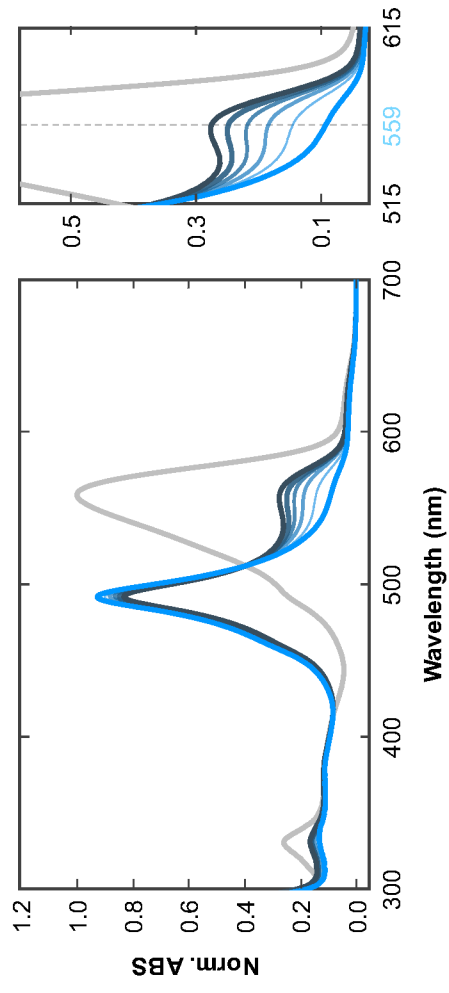
**A** AM1\_1499g1\_S<sub>118</sub>C



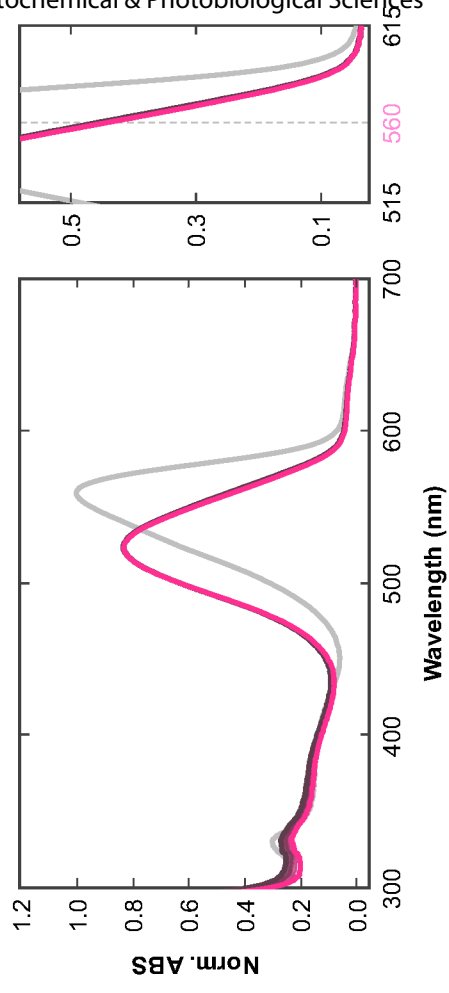
**B** AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C



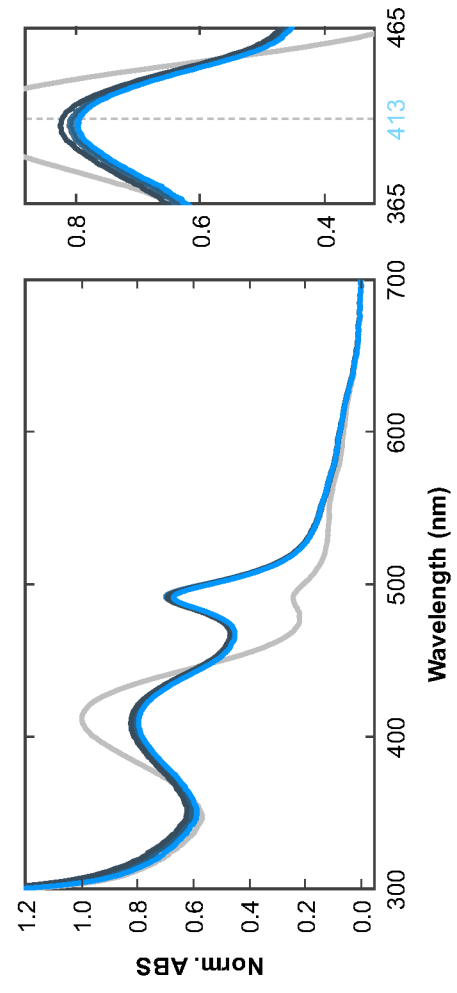
**C** AM1\_1499g1\_S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N



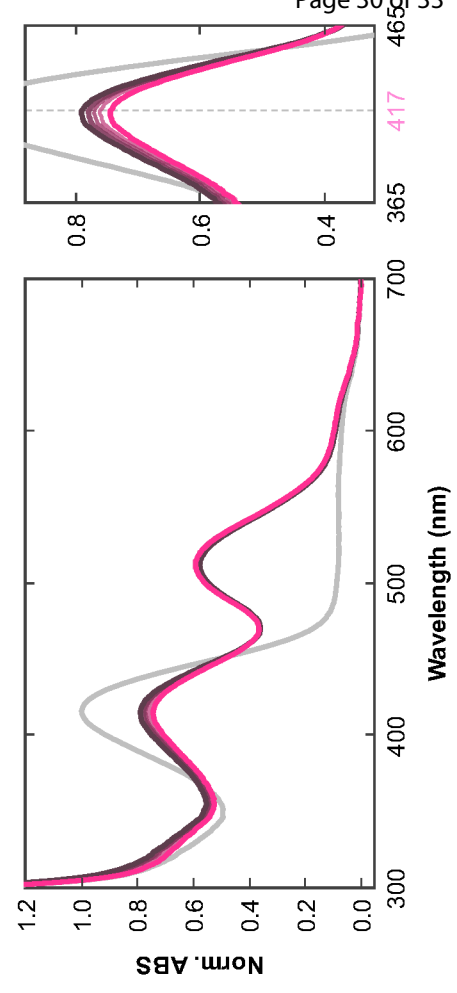
**D** AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C/Y<sub>151</sub>L/T<sub>159</sub>N

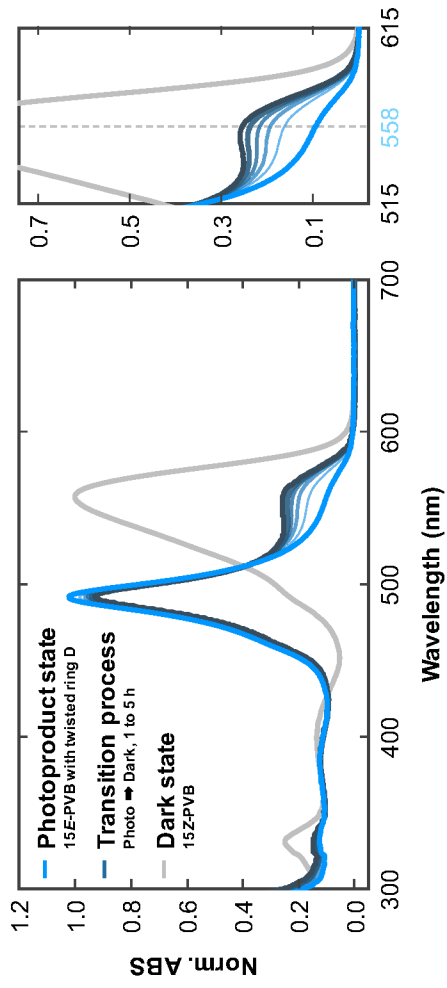
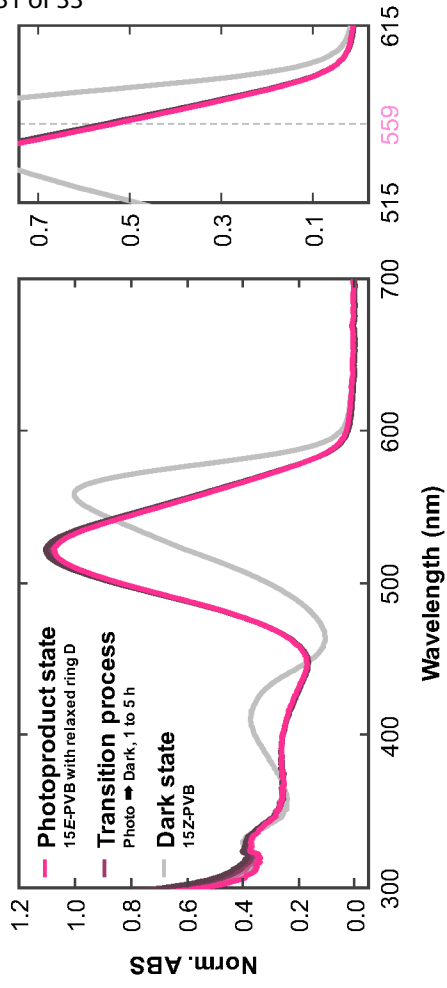
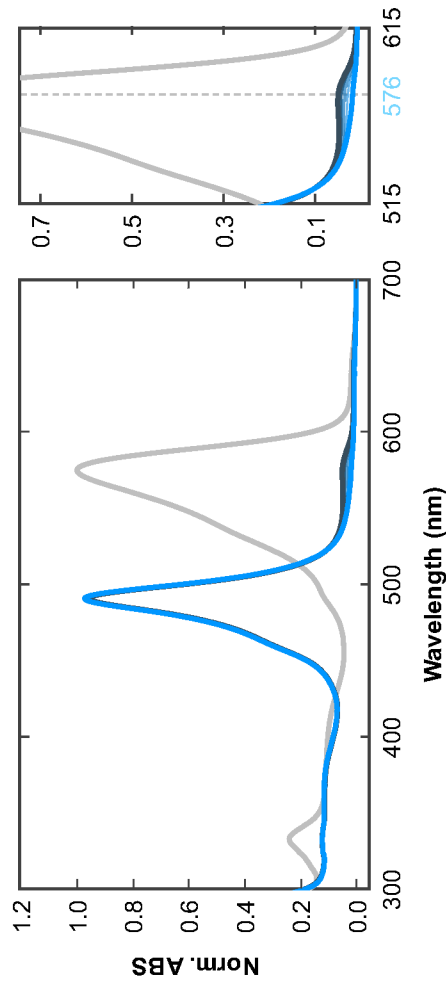
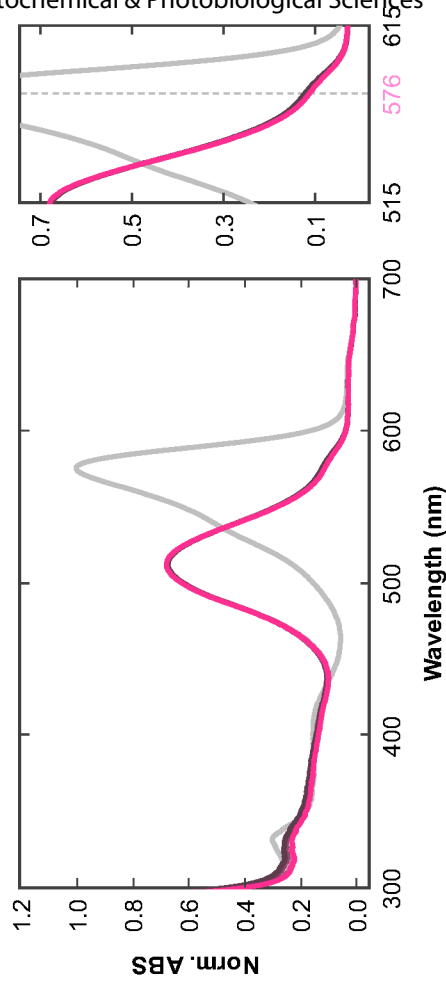


**E** AM1\_1499g1\_S<sub>118</sub>C/H<sub>147</sub>Y

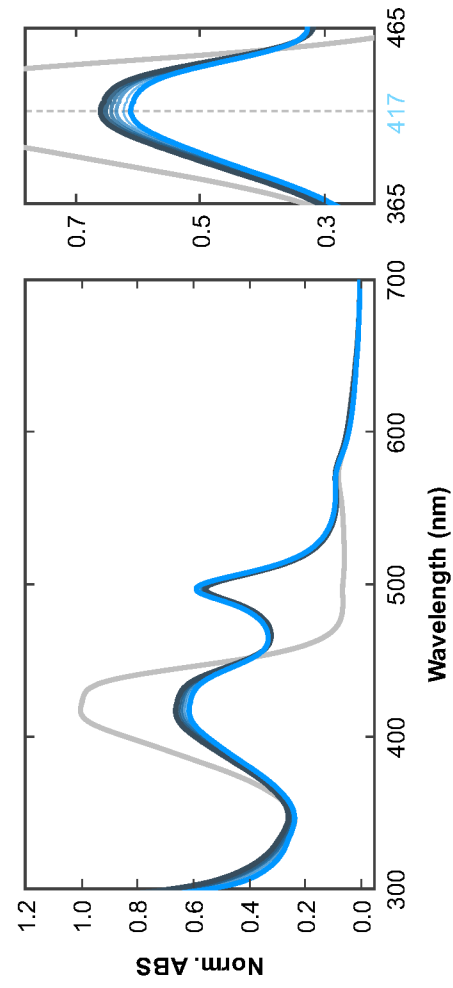
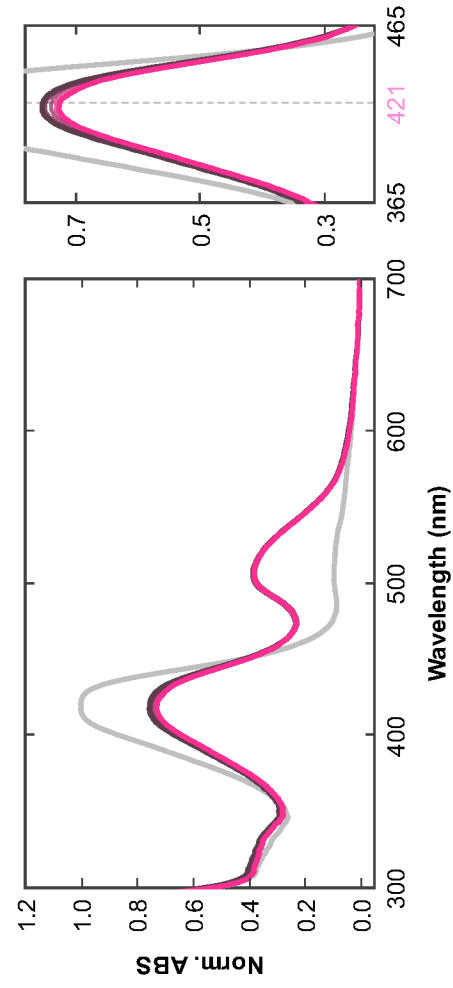


**F** AM1\_1499g1\_F<sub>97</sub>V/S<sub>118</sub>C/H<sub>147</sub>Y

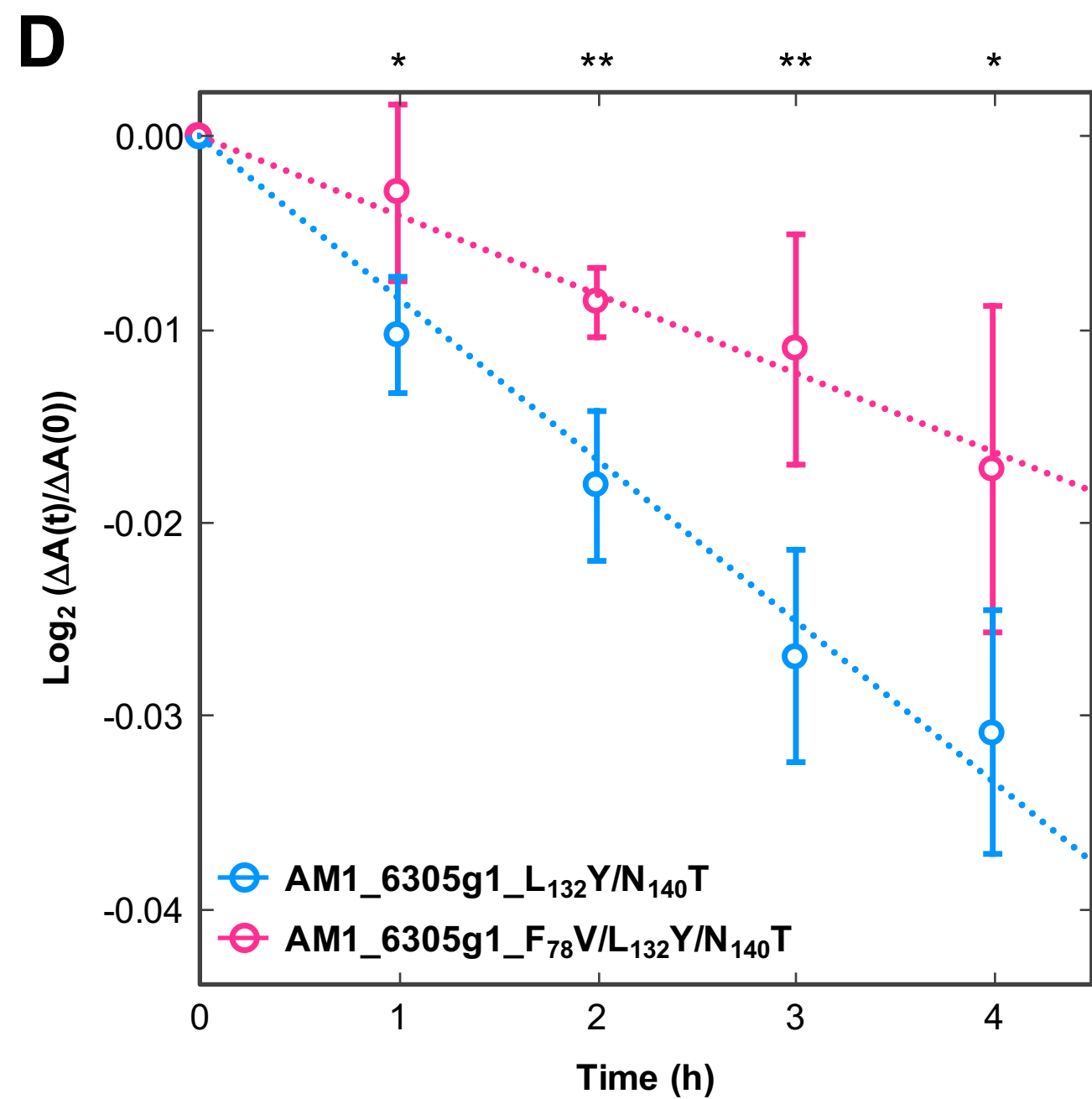
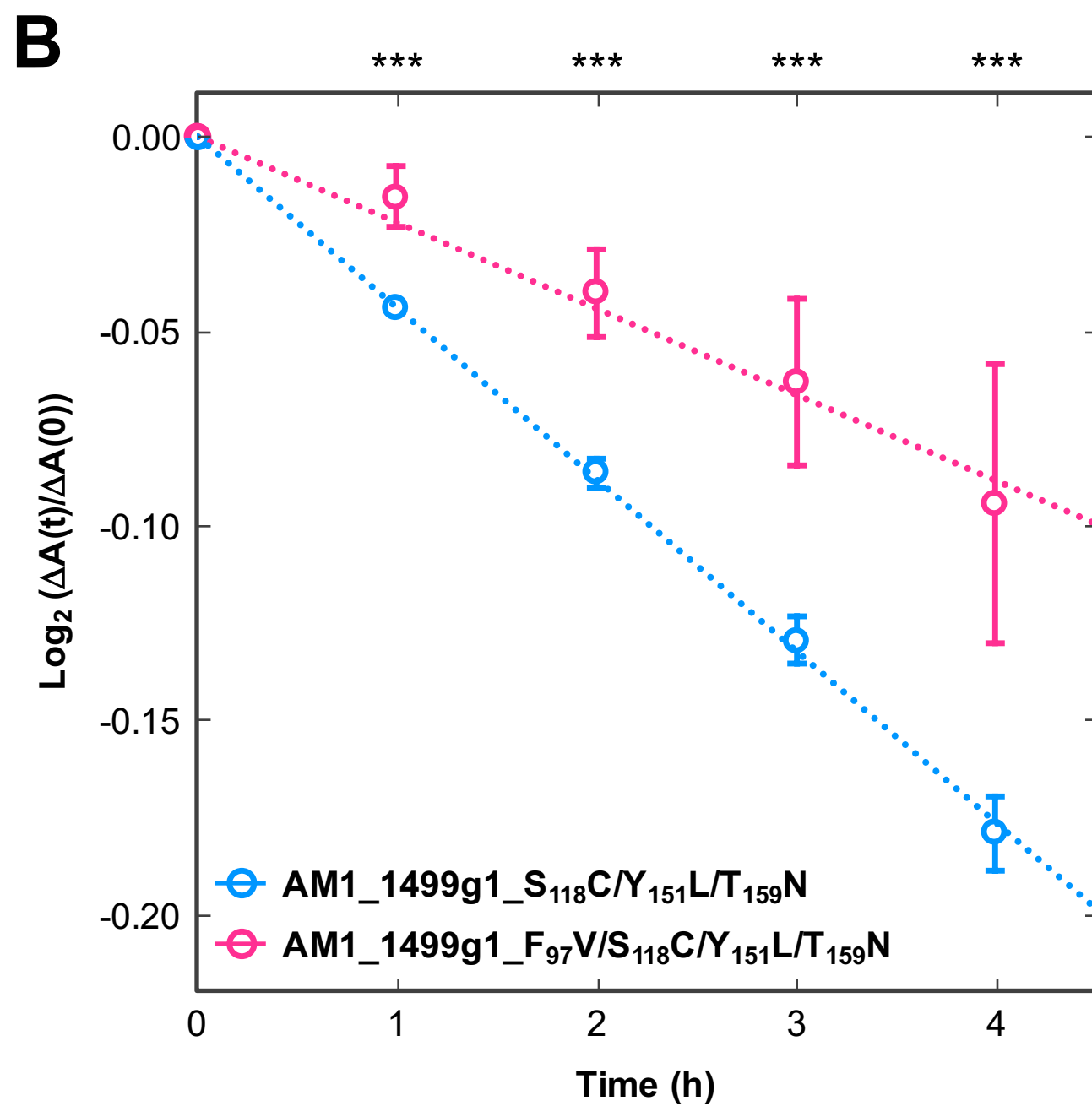
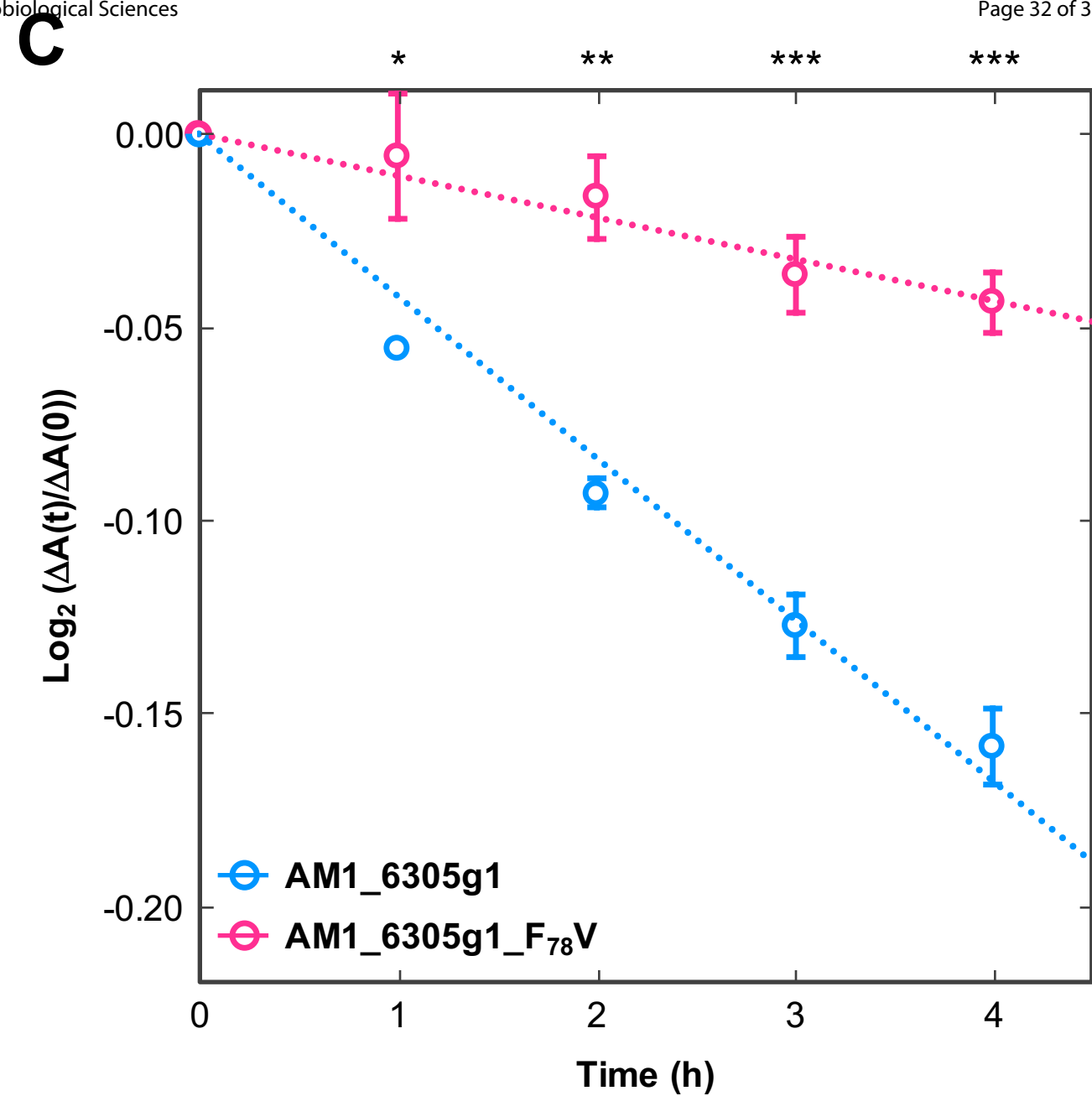
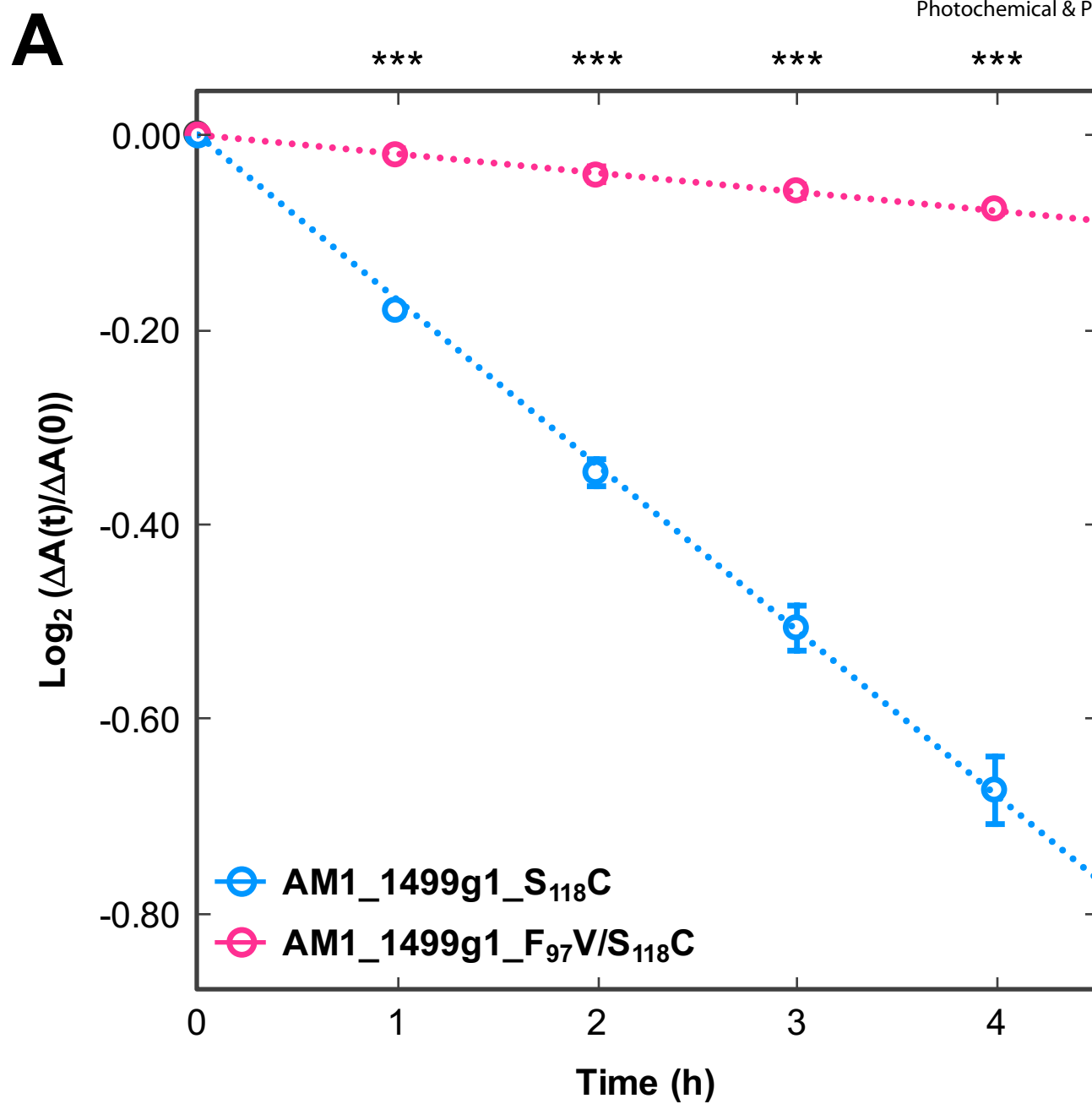


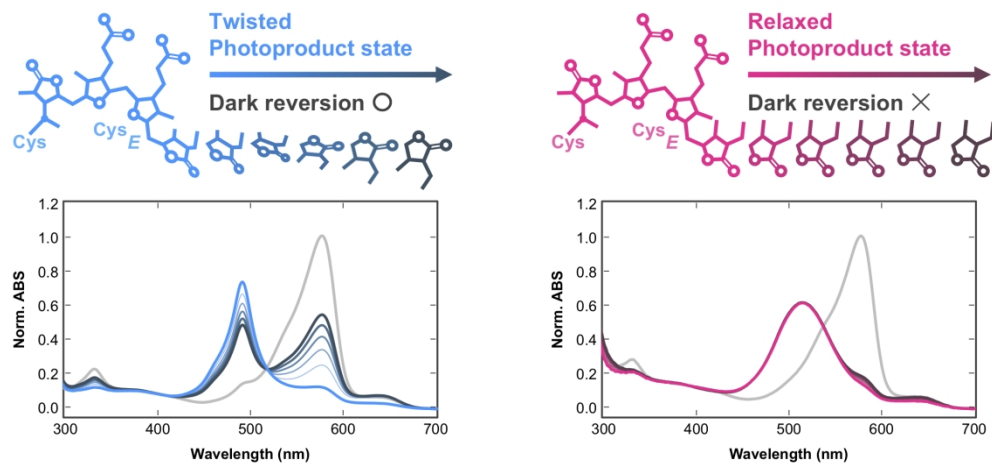
**A** AM1\_6305g1**B** AM1\_6305g1\_F<sub>78</sub>V**C**AM1\_6305g1\_L<sub>132</sub>Y/N<sub>140</sub>T**D**AM1\_6305g1\_F<sub>78</sub>V/L<sub>132</sub>Y/N<sub>140</sub>T**E**

AM1\_0048g1

**F**AM1\_0048g1\_F<sub>241</sub>V/F<sub>298</sub>L







380x177mm (150 x 150 DPI)