Unusual ring D fixation by three crucial residues promotes phycoviolobilin formation in the DXCF-type cyanobacteriochrome without the second Cys

Keiji Fushimi\textsuperscript{1,2,*}, Rei Narikawa\textsuperscript{1,2,3,*}

\textsuperscript{1}Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, Japan
\textsuperscript{2}Core Research for Evolutional Science and Technology, Japan Science and Technology
\textsuperscript{3}Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, Japan

*e-mail: narikawa.rei@shizuoka.ac.jp
Abstract
Cyanobacteriochromes are linear tetrapyrrrole-binding photoreceptors produced by cyanobacteria. Their chromophore-binding GAF domains are categorized into many lineages. Among them, dual Cys-type cyanobacteriochrome GAF domains possessing not only a highly conserved “first Cys” but also a “second Cys” are found from multiple lineages. The first Cys stably attaches to C3¹ of the A-ring, while the second Cys mostly shows reversible ligation to the C10 of the chromophore. Notably, position of the second Cys in the primary sequence is diversified, and the most abundant dual Cys-type GAF domains have a “second Cys” within the DXCF motif, which are called DXCF GAF domains. It has been long known that the second Cys in the DXCF GAF domains not only shows the reversible ligation but also is involved in isomerization activity (reduction of C4=C5 double bond) from the initially incorporated phycocyanobilin to phycoviolobilin. However, comprehensive site-directed mutagenesis on the DXCF GAF domains, AM1_6305g1 and AM1_1499g1, revealed that the second Cys is dispensable for isomerization activity, in which three residues participate by fixing the C- and D-rings. Fixation of the chromophore on both sides of the C5 bridge is necessary, even though one side of the fixation site is far from this bridge, with the other side at C3¹ fixed by the first Cys.
Introduction

Cyanobacteriochromes (CBCRs) are linear tetapyrrole (bilin) -binding photoreceptors that, to date, have been found only in cyanobacteria [1]. The CBCRs are distantly related to red/far-red reversible phytochromes. Only the GAF domain of CBCRs is needed for chromophore incorporation. Phycocyanobilin (PCB) and phycoviolobilin (PVB) are the major chromophores of the CBCR GAF domains (Fig. S1A, B)[2–4], whereas biliverdin and $18^1,18^2$-dihidrobiliverdin can serve as the functional chromophores for some CBCR GAF domains [5–7]. A conserved Cys, called the “first Cys” or “canonical Cys,” in the CBCR GAF domain forms a covalent bond with the C3 side chain of the linear tetapyrrole chromophore via a thioether linkage. The CBCR GAF domains’ light-sensing characteristics depend on the protein and chromophore structures, and absorption of a specific wavelength of light induces $Z/E$ isomerization at the C15=C16 double bond to trigger reversible photoconversion between a 15$Z$ dark state and a 15$E$ photoproduct state (Fig. S1A, B)[1].

The CBCR GAF domains are highly diversified and categorized into many lineages, and consequently, they have various spectral properties.

Of the diverse CBCR GAF domains derived from multiple lineages, DXCF CBCR GAF domains have another conserved Cys, called the “second Cys,” within the Asp-Xaa-Cys-Phe (DXCF) motif [8–19]. Typical DXCF CBCR GAF domains initially incorporate PCB as a precursor chromophore, which is then isomerized to PVB (PCB-to-PVB isomerization activity)[4,20]. The second Cys contributes to this isomerization activity. In addition, the second Cys reversibly attaches to or detaches from the C10 position of PVB during the photoconversion process (i.e., reversible ligation activity)[10,20–23]. In this context, the second Cys residue possesses dual functions involved in isomerization activity and reversible ligation activity.
In our previous study, we firstly identified a novel CBCR GAF domain, AM1_1499g1, which lacks the second Cys, although the domain belongs to a specific DXCF CBCR lineage (AM1_1499g1/AM1_6305g1 lineage) [24]. AM1_1499g1 contains PCB and exhibits an orange/green photocycle but lacks isomerization activity and reversible ligation activity (Fig. S1C). Furthermore, we have succeeded in evolution-inspired color-tuning based on the AM1_1499g1 scaffold; the introduction of the second Cys (S118C variant) results in the acquisition of isomerization activity but not reversible ligation activity, with the exhibition of a yellow/teal photocycle (Fig. S1C). In addition, replacement of Tyr and Thr near the D-ring of the chromophore with Leu and Asn, respectively, (S118C/Y151L/T159N variant, based on the S118C variant molecule) yields a dark state blue-shift, likely due to the D-ring twist (Fig. S1D, E), with the molecule subsequently displaying a green/teal photocycle (Fig. S1C). Furthermore, replacement of the His next to the first Cys residue with Tyr (S118C/H147Y variant) enables reversible ligation activity, in which the bulky side chain may push the C10 bridge between the B-ring and C-ring toward the second Cys to facilitate reversible ligation (Fig. S1D, E) with the presentation of a blue/teal photocycle (Fig. S1C).

On the other hand, AM1_6305g1, a close homolog of AM1_1499g1, retains the second Cys, and displays a green/teal photocycle [15]. AM1_6305g1 can isomerize PCB to PVB but cannot ligate the second Cys to the C10 position. We have performed reverse engineering on AM1_6305g1, based on the engineering work of AM1_1499g1, and succeeded in developing two variant molecules showing yellow/teal and blue/teal photocycles for modification of the dark-state D-ring twist and reversible Cys ligation [24]. However, the replacement of the second Cys with Ser unexpectedly did not affect the isomerization activity. Starting from this unanticipated observation, we identified three residues key for the isomerization activity in addition to the second Cys in this study. These findings provide a general concept for the isomerization mechanism.
Results and discussion

Second Cys residue is dispensable for PCB-to-PVB isomerization in the AM1_6305g1 scaffold

We have previously reported engineering of the CBCR GAF domains, AM1_1499g1 and AM1_6305g1, within one of the DXCF lineages, AM1_1499g1/AM1_6305g1 lineage [24]. AM1_1499g1 lacks the second Cys showing neither isomerization activity nor reversible ligation activity, while AM1_6305g1 has the second Cys showing only isomerization activity but not reversible ligation activity. The introduction of the second Cys in AM1_1499g1, as part of the primary engineering phase, resulted in the acquisition of isomerization activity but not reversible ligation activity (Fig. S1C). This result encouraged us to perform reverse engineering on AM1_6305g1 for loss of the isomerization activity.

We thus replaced the second Cys of AM1_6305g1 with Ser, the original residue in AM1_1499g1 (AM1_6305g1_C99S) (Fig. S2A-i). Unexpectedly, AM1_6305g1_C99S showed a green/teal photocycle, which is nearly the same as that of the wild-type (Fig. 1A, Fig. 2A, and Table 1). Furthermore, the chromophore incorporated into AM1_6305g1_C99S was assigned not to PCB but to PVB by comparing the denatured difference absorption spectrum (dark state–photoproduct state) of the C99S variant with those of the PCB- and PVB-binding CBCR GAF domains (Fig. 3A and Table 1). These results indicate that the second Cys residue is dispensable for the isomerization activity in the AM1_6305g1 scaffold. Ser and Cys have a common skeleton with distinctive functional groups at the end of the side-chains; a hydroxy group for Ser and a thiol group for Cys. We speculate that the hydroxy group of Ser can complement the functionality of the thiol group of Cys in AM1_6305g1.

We next replaced these polar amino acids with Ala (AM1_6305g1_C99A), which has a nonpolar methyl group smaller than that of Ser and Cys to verify this assumption (Fig. S2A-ii). Spectral analysis revealed two photoconvertible components for...
AM1_6305g1_C90A; green/teal and orange/green (Fig. 4A). We were able to separately excite a single component and independently characterize the photoconversion properties and the binding chromophore species based on the absorption spectra of the native and denatured molecules using various monochromic light sources (Fig. 4B, C and Table 1). The green/teal component bound PVB (Fig. 4C and Table 1, shown by a cyan line) and showed reversible photoconversion between a green-absorbing dark state (~560 nm) and a teal-absorbing photoproduct state (~490 nm) (Fig. 4A, shown by cyan arrowheads). The orange/green component bound PCB (Fig. 4C and Table 1, shown by a magenta line) and showed reversible photoconversion between an orange-absorbing dark state (~620 nm) and a green-absorbing photoproduct state (~550 nm) (Fig. 4A, shown by magenta arrowheads). Our findings taken together indicate that AM1_6305g1_C90A is a mixture of PCB- and PVB-binding populations.

In conclusion, the second Cys residue is dispensable for the isomerization activity in the AM1_6305g1 scaffold. Ser, but not Ala, could complement the function of the Cys residue in the AM1_6305g1 scaffold. Furthermore, the fact that AM1_6305g1_C90A showed partial isomerization activity indicates the contribution of another residue(s) to the isomerization activity.

Leu132 and Asn140 in AM1_6305g1 are crucial for efficient PCB-to-PVB isomerization without the second Cys residue

We have found that the AM1_6305g1 scaffold is clearly distinct from the AM1_1499g1 scaffold in the context of isomerization activity, despite their close homologous relationship. Namely, the second Cys residue is dispensable for isomerization activity in the AM1_6305g1 scaffold but not in the AM1_1499g1 scaffold, indicating that the distinct residues between these two molecules are determinants for this divergence. We have already determined in a
previous study that such amino acid alterations are crucial for color-tuning of the dark state via a D-ring twist; Leu\textsubscript{132}/Asn\textsubscript{140} in AM1\_6305g1 and Tyr\textsubscript{151}/Thr\textsubscript{159} in AM1\_1499g1 (Fig. S1C–E)[24]. We hypothesize that the alterations of these two residues are key not only for the dark-state color-tuning but also for isomerization activity. Although replacement of Leu\textsubscript{132}/Asn\textsubscript{140} with Tyr/Thr based on the AM1\_6305g1 wild-type background (AM1\_6305g1\_L\textsubscript{132}Y/N\textsubscript{140}T) resulted in a red shift of the dark state, no effects on the isomerization activity have been detected (Fig. 1B-i, Fig. 2B-i, Fig. 3B-i, and Table 1)[24]. In other words, this variant molecule has a single yellow/teal photoconvertible component, and the binding chromophore species of this variant molecule are composed of PVB as well as the wild-type. In this context, these two residues may be crucial for the isomerization activity without the second Cys residue.

To verify this hypothesis, we replaced these two residues (Leu\textsubscript{132} and Asn\textsubscript{140}) with Tyr and Thr, respectively, based on the C\textsubscript{99}S background molecule (AM1\_6305g1\_C\textsubscript{99}S/L\textsubscript{132}Y/N\textsubscript{140}T) (Fig. S2A-i). AM1\_6305g1\_C\textsubscript{99}S/L\textsubscript{132}Y/N\textsubscript{140}T had two photoconvertible components displaying yellow/teal and orange/green photocycles, in contrast to the L\textsubscript{132}Y/N\textsubscript{140}T variant (Fig. 1B-ii). The difference spectra of these two components were similar to those of the PVB-binding and PCB-binding reference molecules showing yellow/teal and orange/green photocycles, respectively (Fig. 2B-ii and Table 1). The chromophores incorporated into these photoconvertible components, the yellow/teal and orange/green ones of AM1\_6305g1\_C\textsubscript{99}S/L\textsubscript{132}Y/N\textsubscript{140}T, were assigned to PVB and PCB, respectively, based on their denatured difference absorption spectra (Fig. 3B-ii and Table 1). We could not obtain a pure difference spectrum of the denatured yellow/teal component because there was much less of the yellow/teal component than the orange/green one. However, a double-difference spectrum (normalized difference spectrum\textsubscript{yellow/teal component}− normalized difference spectrum\textsubscript{orange/green component}) clearly showed that the yellow/teal...
component was derived from PVB incorporation (Fig. 3B-ii and Table 1, shown by a violet line). These results suggested that the Ser residue itself could not fully complement the function of the second Cys residue and that the Leu and Asn residues, but not the Tyr and Thr residues, could support the Ser residue for full isomerization of PCB to PVB (Fig. 1A, B, Fig. 2A, B, Fig. 3A, B, and Table 1). Thus, these two amino acid positions are involved not only in dark-state color-tuning but also in isomerization activity.

We constructed singly mutated variant molecules based on the C$_{99}$S background molecule (AM1_6305g1_C$_{99}$S/L$_{132}$Y and C$_{99}$S/N$_{140}$T) to elucidate the contribution of each residue to the isomerization activity (Fig. S2A-iii). These two variant molecules had yellow/teal and orange/green components, as well as the doubly mutated ones (AM1_6305g1_C$_{99}$S/L$_{132}$Y/N$_{140}$T) (Fig. S3A, B). A large amount of yellow/teal component was detected for the C$_{99}$S/L$_{132}$Y variant, whereas a smaller amount of the same component was detected for the C$_{99}$S/N$_{140}$T variant; the contribution of the residues at the Leu/Tyr position to the isomerization activity was larger than that of the Asn/Thr position.

The PVB-binding dark state of the AM1_6305g1_C$_{99}$S/L$_{132}$Y/N$_{140}$T, the yellow-absorbing form, was red-shifted in comparison with that of the C$_{99}$S background molecule, which is consistent with the same L$_{132}$Y/N$_{140}$T replacement on the wild-type background (Fig. 1A, B, Fig. 2A, B, and Table 1). This finding could be explained by the previously proposed mechanism, in which replacement with Tyr and Thr resulted in the cancellation of the D-ring twist, leading to the red shift [24]. On the other hand, because the C$_{99}$S background molecule did not contain any PCB-binding component, we could not discuss the dark-state color-tuning of the PCB-binding ones (Fig. 2A-ii and Table 1).

In conclusion, the Ser residue can fully complement the isomerization function of the second Cys residue only when the Leu and Asn residues hold the D-ring with twisted geometry. However, the AM1_6305g1_C$_{99}$S/L$_{132}$Y/N$_{140}$T variant still contained PVB-
binding yellow/teal components, albeit at lower levels, indicating the additional contribution of another residue(s).

Gln<sub>112</sub> in AM1_6305g1 is a third key residue for isomerization activity in the absence of the second Cys residue

In the previous section, we focused on the Leu/Tyr and Asn/Thr positions in the context of residues specific to AM1_1499g1 among the molecules within the AM1_1499g1/AM1_6305g1 lineage (Fig. S1E). We next focused on the residue(s) specific to AM1_6305g1 near the chromophore among the molecules of this lineage and found the Gln/Arg position near the C-ring propionate. AM1_6305g1 specifically possesses a Gln residue (Gln<sub>112</sub>) at this position, whereas other molecules within this lineage contain Arg residues (Fig. S1D, E). We constructed four variants (AM1_6305g1<sub>Q<sub>112</sub>R</sub>, C<sub>99S/Q<sub>112</sub>R</sub>, Q<sub>112</sub>R/L<sub>132</sub>Y/N<sub>140</sub>T, and C<sub>99S/Q<sub>112</sub>R/L<sub>132</sub>Y/N<sub>140</sub>T) based on their corresponding background molecules (AM1_6305g1 wild-type, C<sub>99S</sub>, Л<sub>132</sub>Y/N<sub>140</sub>T, and C<sub>99S/L<sub>132</sub>Y/N<sub>140</sub>T), respectively, to elucidate the function of the Gln residue (Fig. S2A-i). A single green/teal component was detected for the Q<sub>112</sub>R and C<sub>99S/Q<sub>112</sub>R</sub> variants as well as their background molecules (wild-type and C<sub>99S</sub>) (Fig. 1C, Fig. 2C, and Table 1), whereas a single yellow/teal component was detected for the Q<sub>112</sub>R/L<sub>132</sub>Y/N<sub>140</sub>T variant as well as its background molecule (L<sub>132</sub>Y/N<sub>140</sub>T) (Fig. 1D-i, Fig. 2D-i, and Table 1). The chromophores incorporated into these variant molecules were assigned to PVB, indicating that they retained full isomerization activity (Fig. 3C, 3D-i, and Table 1). Conversely, the C<sub>99S/Q<sub>112</sub>R/L<sub>132</sub>Y/N<sub>140</sub>T variant molecule had close to a single orange/green component with a faint yellow/teal component, which corresponded to the PCB- and PVB-binding populations, respectively (Fig. 1D-ii, Fig. 2D-ii, Fig. 3D-ii, and Table 1). In conclusion, the replacement of a total of four residues (C<sub>99S/Q<sub>112</sub>R/L<sub>132</sub>Y/N<sub>140</sub>T) resulted in almost complete inactivation of the isomerization activity.
Summary of engineering on AM1_6305g1

Based on the sequence comparison, we have identified four key residues (Cys$_{99}$/Gln$_{112}$/Leu$_{132}$/Asn$_{140}$) of the AM1_6305g1 involved in the isomerization activity from PCB to PVB (Fig. S1D, E). Only the second Cys (Cys$_{99}$) is sufficient for full isomerization among these residues, while any replacement of the other residues (Gln$_{112}$/Arg, Leu$_{132}$/Tyr and Asn$_{140}$/Thr) under the presence of Cys$_{99}$ did not at all affect the isomerization activity (Fig. 1A-i to D-i, Fig. 2A-i to D-i, and Table 1). Leu$_{132}$ and Asn$_{140}$ plays a central role in isomerization activity under the presence of a Ser in place of the second Cys, while Gln$_{112}$ plays a supportive role (Fig. 1A-ii to D-ii, Fig. 2A-ii to D-ii, Fig. 3A-ii to D-ii, and Table 1). Namely, AM1_6305g1 has constructed a robust system for isomerization activity, in which Gln$_{112}$, Leu$_{132}$ and Asn$_{140}$ can support the Ser residue, substitute of the second Cys.

Reverse engineering on AM1_1499g1

In contrast to AM1_6305g1, AM1_1499g1 has Ser$_{118}$/Arg$_{131}$/Tyr$_{151}$/Thr$_{159}$ residues corresponding to Cys$_{99}$/Gln$_{112}$/Leu$_{132}$/Asn$_{140}$ of AM1_6305g1 (Fig. S1D, E). This fact suggests that AM1_1499g1 has completely lost the redundancy system for the isomerization activity observed for AM1_6305g1. To verify this hypothesis, we performed reverse engineering on AM1_1499g1 to generate variant molecules (Y$_{151}$/L/T$_{159}$N, R$_{131}$/Q, R$_{131}$/Q/Y$_{151}$/L/T$_{159}$N, S$_{118}$/C, S$_{118}$/C/Y$_{151}$/L/T$_{159}$N, S$_{118}$/C/R$_{131}$/Q, and S$_{118}$/C/R$_{131}$/Q/Y$_{151}$/L/T$_{159}$N) based on the wild-type molecule corresponding to each AM1_6305g1 variant (Fig. S2B-i) and compared each (Figs. 5–7, and Table 1). All variant molecules possessing the second Cys (S$_{118}$/C, S$_{118}$/C/Y$_{151}$/L/T$_{159}$N, S$_{118}$/C/R$_{131}$/Q, and S$_{118}$/C/R$_{131}$/Q/Y$_{151}$/L/T$_{159}$N) incorporated only PVB and showed yellow/teal or green/teal reversible photoconversion (Fig. 5A-i to D-i, Fig. 6A-i to D-i, and Table 1). This result is fully consistent with the case of
AM1_6305g1; the second Cys is also sufficient for the isomerization activity for the AM1_1499g1 scaffold. Variants possessing Tyr\textsubscript{151}/Thr\textsubscript{159} showed a yellow/teal photocycle, while variants possessing Leu\textsubscript{151}/Asn\textsubscript{159} showed a green/teal photocycle, which is well explained by the dark-state trapped twist model [24].

Under the absence of the second Cys, the phenotypes of the variant molecules were partially inconsistent with the case of AM1_6305g1, particularly in two respects (Fig. 5A-ii to D-ii, Fig. 6A-ii to D-ii, Fig. 7A-ii to D-ii, and Table 1). First, Leu\textsubscript{151}, Asn\textsubscript{159}, and Gln\textsubscript{131} without the second Cys (R\textsubscript{131}Q/Y\textsubscript{151}L/T\textsubscript{159}N) displayed only partial isomerization activity, harboring both the PCB-binding orange/green component and the PVB-binding green/teal component (Fig. 5A-ii, Fig. 6A-ii, 7A-ii, and Table 1). Second, Gln\textsubscript{131} had almost no contribution to the isomerization activity because the R\textsubscript{131}Q variant did not show detectable isomerization activity compared to the wild-type (Fig. 5B-ii and D-ii, Fig. 6B-ii and D-ii, 7B-ii and D-ii, and Table 1). Conversely, the functions of the Tyr\textsubscript{151} and Thr\textsubscript{159} positions were consistent with the case for AM1_6305g1. Namely, Y\textsubscript{151}L/T\textsubscript{159}N replacement based on the wild-type and R\textsubscript{131}Q backgrounds resulted in the restoration of partial, but not complete, isomerization activity (Fig. 5A-ii and C-ii, Fig. 6A-ii and C-ii, 7A-ii and C-ii, and Table 1). Characterization of the singly mutated variants (Y\textsubscript{151}L and T\textsubscript{159}N) based on the wild-type revealed that the Y\textsubscript{151}L replacement had a greater effect on the isomerization activity than the T\textsubscript{159}N substitute (Fig. S3C, D). The Y\textsubscript{151}L replacement resulted in a significant promotion of isomerization, whereas the T\textsubscript{159}N replacement had a subtle effect on isomerization activity. This differential contribution is consistent with the case of the AM1_6305g1 scaffold (Fig. S3A, B). The dark-state color-tuning is also applicable to the PCB-binding molecules, based on comparison of the difference spectra of the PCB-binding components between the wild-type and Y\textsubscript{151}L/T\textsubscript{159}N molecules; the dark state of the wild-type possessing Tyr\textsubscript{151} and Thr\textsubscript{159} was red-shifted in comparison with that of the Y\textsubscript{151}L/T\textsubscript{159}N variant molecule possessing
Leu₁₅₁ and Asn₁₅₉ (Fig. 6A-ii, 6C-ii and D-ii, and Table 1). The R₁₃₁Q variant showed a temperature-dependent spectral shift without chromophore isomerization, similar to the wild-type; photoconversion between an orange-absorbing form peaking at 619 nm and a green-absorbing form peaking at 542 nm was observed at a low temperature (5°C), whereas photoconversion between the orange-absorbing form peaking at 628 nm and the green-absorbing form peaking at 545 nm was observed at a high temperature (30°C) (Fig. S4)[24].

**Possible mechanism of the PCB-to-PVB isomerization**

To begin, we discuss the possible PCB-to-PVB isomerization mechanism of the typical DXCF CBCR GAF domains showing blue/green reversible photoconversion, such as TePixJg. A previous reconstitution study has revealed the reaction flow of chromophore incorporation and isomerization [20]. PCB is initially incorporated into the protein pocket, and the first Cys covalently binds to C3\(^1\) position of the chromophore by self-ligation activity (Fig. 8A-i). The second Cys ligates to the C10 position of the chromophore in the dark state prior to isomerization from PCB to PVB after chromophore incorporation (Fig. 8A-ii). At this point, blue/green reversible photoconversion has already been observed, albeit with PCB incorporation, suggesting that the C4=C5 double bond of PCB between the A- and B-rings is highly distorted. Furthermore, the C10 bridge between the B- and C-rings would be very bent by the second Cys ligation based on structural information of the PVB-binding TePixJg. As a result, the high distortion of the C4=C5 double bond was stably established by dual fixation of the chromophore at the A-ring and C10 position via first and second Cys residues. Such a highly unstable and rigid conformation would promote the reduction of the C4=C5 double bond and concomitant oxidation of the C2–C3 single bond of the A-ring by a reaction model proposed by Rockwell et al. [13]. After a series of reactions, PCB is finally isomerized to PVB.
On the other hand, although the green/teal CBCR GAF domains such as AM1_6305g1 do not form covalent bond formation between the second Cys and the C10 position of the chromophore in both absorbing forms (Fig. 8A-iii)[15], these domains retain the isomerization activity. Therefore, these domains can display the isomerization activity without covalently fixing the C10 of the chromophore. In this context, distortion of the C4=C5 double bond would be established in an alternative way. In this study, we determined that three residues of AM1_6305g1 (Gln₁₁₂, Leu₁₃₂, and Asn₁₄₀) contributed to isomerization activity when the second Cys was replaced with Ser (Fig. 1A-ii to D-ii, Fig. 2A-ii to D-ii, Fig. 3A-ii to D-ii, and Table 1). It is of note that replacements of these residues do not largely affect chromophore-binding efficiency and photoconversion capability, suggesting specific contribution to the isomerization activity. Conversely, these residues are dispensable under the presence of the second Cys (Fig. 1A-i to D-i, Fig. 2A-i to D-i, Fig. 3A-i to D-i, and Table 1). The second Cys, but not the Ser, can completely hold the chromophore in the C4=C5 distorted conformation. The thiol group of the second Cys may have a unique electrostatic interaction with the chromophore near the C10 position, which could not be fully complemented by the hydroxy group of the Ser.

Under the absence of the second Cys, Gln₁₁₂/Leu₁₃₂/Asn₁₄₀ residues in addition to Ser₉₉, cooperatively work to produce the isomerization activity (Fig. 8A-iv). Ser₉₉ may have a weaker interaction with the chromophore than the second Cys, and these three residues would support the function of Ser₉₉. The Leu₁₃₂/Asn₁₄₀ residues have a larger contribution than the Gln₁₁₂ residue (Fig. 1A-ii to D-ii, Fig. 2A-ii to D-ii, Fig. 3A-ii to D-ii, and Table 1). The Leu₁₃₂/Asn₁₄₀ residues have a larger contribution than the Gln₁₁₂ residue (Fig. 1A-ii to D-ii, Fig. 2A-ii to D-ii, Fig. 3A-ii to D-ii, and Table 1). The Leu₁₃₂/Asn₁₄₀ residues have been elucidated to fix the D-ring into a twisted geometry, which would assist the Ser₉₉ in the distortion of the C4=C5 double bond. Gln₁₁₂ is predicted to be positioned near the C-ring...
propionate and contribute to the stabilization of the chromophore conformation. However, this prediction is based on the TePixJg structure with the “bent” chromophore, whose conformation would be largely different from that of “unbent” one (Fig. S1B, D). Therefore, it is difficult to elucidate the precise role of Gln\textsubscript{112} in AM1\textsubscript{6305}g1. It is of note that not only does the second Cys have no direct interactions with the C4=C5 double bond, but the three residues also do not. In particular, these three residues would have interaction with the chromophore around the C- and D-rings far from the C4=C5 bond. Fixation of the chromophore on both sides of the C5 bridge between the A- and B-rings would be important for isomerization activity, even though one side of the fixation site is far from this bridge with another side fixed at C3\textsuperscript{1} by the first Cys. Such a double fixed situation resulted in high distortion of the C4=C5 double bond with great tension. Protonation at C4 and C5 may occur to cancel the tension, which would be a trigger for isomerization. Although it is difficult to predict the proton donor, Leu\textsubscript{132}, Asn\textsubscript{140} and is Gln\textsubscript{112}, which are residues far from the C4=C5 bond, are not likely to be directly involved in the protonation process. Detailed spectroscopic analyses should be needed to elucidate the protonation process. Although no naturally occurring molecules showing isomerization activity in the absence of the second Cys have been reported, this study provides the potential for the presence of such natural molecules that apply a remote fixation system.

**Evolutionary trace of the AM1\textsubscript{1499}g1/AM1\textsubscript{6305}g1 lineage**

We constructed a phylogenetic tree of the CBCR GAF domains with the phytochrome GAF domains as the outgroup for the evolutionary trace (Fig. 8B-i). The DXCF CBCR GAF domains are detected in several lineages, and the AM1\textsubscript{1499}g1/AM1\textsubscript{6305}g1 lineage is one of such families. This lineage shares an ancestor with the expanded red/green (XRG) lineage
GAF domains. The first branching resulted in two subgroups within this lineage (Fig. 8B-ii). Notably, these two subgroups include green/teal photoconvertible molecules, indicating that this lineage’s ancestral molecule would be the green/teal photoconvertible version showing isomerization activity but not reversible Cys ligation capabilities. Furthermore, molecules of this lineage, except for AM1_1499g1, retain the Leu/Asn residues that largely contribute to the isomerization activity without the second Cys residue (Fig. S1E). We consider that, because the ancestral molecule of this lineage originally lost the ligation ability conferred by the second Cys, the weaker interaction mode of the second Cys has promoted the acquisition of the Leu/Asn residues for robust isomerization activity.

These subgroups are further divided into two populations. Blue/teal photoconvertible molecules showing not only isomerization activity but also reversible Cys ligation have emerged in the case of one subgroup (Fig. 8B-ii). This event would occur with the replacement of the His residue with Tyr next to the first Cys, in which the bulky Tyr side chain may push the C10 bridge to facilitate Cys ligation at the opposite side, based on the previous study (Fig. S1E)[24]. On the other hand, in the case of the other subgroup, AM1_1499g1 has emerged, which has lost the second Cys and does not show isomerization activity (Fig. 8B-ii and Fig. S1E). However, the present study identified that the replacement of only the second Cys is insufficient for the complete inactivation of the isomerization activity. In fact, AM1_1499g1 has lost not only the second Cys but also the Leu/Asn residues (Fig. S1E). AM1_1499g1 has experienced multiple amino acid replacements after diversification to establish a longer wavelength perception (Fig. 8A-v and B). On the other hand, AM1_6305g1 has specifically acquired Gln112, which supports isomerization activity for a more robust isomerization system (Fig. 8B-ii and Fig. S1E). The whole protein domain architectures are quite similar to each other and these proteins are derived from the same organism, Acaryochloris marina, suggesting that these two domains may have evolved in the
opposite direction to acquire dual windows for shorter and longer light perception after the
protein duplication event. In summary, molecules within this lineage have undergone unique
evolutionary events to develop highly diverse green/teal, blue/teal, and orange/green
photocycles through only five amino acid replacements in total.
**Materials and Methods**

**Bacterial strains and growth media**

The *Escherichia coli* strain JM109 (TaKaRa) was used for plasmid DNA cloning. The *E. coli* strain C41 (Cosmo Bio) harboring plasmid pKT271 (constructed by heme oxygenase and phycocyanobilin:ferredoxin oxidoreductase genes from *Synechocystis* sp. PCC 6803)\[25\] was used for co-expression of proteins with the PCB-producing system. Bacterial cells were grown on lysogeny broth (LB) agar medium containing 20 µg/mL kanamycin with or without 20 µg/mL chloramphenicol for selection of plasmid-transformed cells.

**Bioinformatics**

Multiple sequence alignment and neighbor-joining phylogenetic trees were constructed using MEGA7 software \[26\]. The crystal structure of PVB–bound TePixJg (blue-absorbing form, PDB_ID: 4GLQ) was utilized to assess key amino acid residues for isomerization. The molecular graphic was generated using the UCSF Chimera software \[27\].

**Plasmid construction**

AM1_6305g1 (amino acid positions 33–203)\[15\] and AM1_1499g1 (amino acid positions 47–222)\[24\] genes fused with an N-terminal His-tag sequence have been inserted into a pET28a vector (Novagen) for protein expression in *E. coli*. The plasmids of AM1_6305g1 and AM1_1499g1 variants were generated by site-directed mutagenesis using each parent plasmid. KOD One PCR Master Mix (Toyobo Life Science) with the appropriate nucleotide primer sets was used for mutagenesis. The primer sets of forward primer GATGATxxxTTTGGTCATAATTATGCCAATAAA and reverse primer ACCAAAyyyATCATCGCTACATTGGC for the replacement of Cys99 of AM1_6305g1 with Ser (xxx = agt, yyy = act) and Ala (xxx = gcc, yyy = ggc) were prepared. The primer set
of forward primer CAAGGAcggGTGGTTGCGGAT and reverse primer AAACACCggTCCTTGGCAGATTTGATGC for the replacement of Gln\textsubscript{112} of AM1\textsubscript{6305g1} with Arg, and conversely, the primer set of forward primer TTGGGGcagGTATTTGCAGTCGGATTGATGT and reverse primer AAATACctgCCCAAATTTGATTTGCGCATATAATT for the replacement of Arg\textsubscript{131} of AM1\textsubscript{1499g1} with Gln were prepared, respectively. The other primer sets for the replacements of Leu\textsubscript{132} of AM1\textsubscript{6305g1} with Tyr, Asn\textsubscript{140} of AM1\textsubscript{6305g1} with Thr, Ser\textsubscript{118} of AM1\textsubscript{1499g1} with Cys, Tyr\textsubscript{151} of AM1\textsubscript{1499g1} with Leu, and Thr\textsubscript{159} of AM1\textsubscript{1499g1} with Asn were constructed in a previous study [24]. All plasmid sequences were confirmed by DNA sequencing (Eurofins Genomics).

### Protein expression and purification

*E. coli* C41 pKT271 was cultured in 1 L LB medium at 37°C until the optical density at 600 nm was 0.4–0.8, and then the cells were cultured overnight at 18°C after isopropyl β-D-1-thiogalactopyranoside (IPTG) addition (final concentration, 0.1 mM) for induction of the protein expression. The cells were collected by centrifugation at 5,000 g for 15 min and were suspended in lysis buffer (20 mM HEPES-NaOH pH 7.5, 0.1 M NaCl, and 10% (w/v) glycerol). All proteins were extracted and purified as described in the previous study [24].

### SDS-PAGE analysis

Purified proteins were diluted in buffer (60 mM Tris–HCl pH 8.0, 2% (w/v) sodium dodecyl sulfate (SDS), and 60 mM dithiothreitol (DTT)), denatured at 95°C for 3 min, and electrophoresed at room temperature (r.t.) using 12% (w/v) SDS polyacrylamide gels. The electrophoresed gels were soaked in distilled water for 30 min and then exposed to blue light (\(\lambda_{max} = 470\) nm) and green light (\(\lambda_{max} = 527\) nm) using a WSE-5500 VariRays (ATTO) with a
short-path filter (passing through < 562 nm) to visualize the fluorescence of the proteins. The fluorescence bands were imaged using a WSE109 6100 LuminoGraph (ATTO) with a long-path filter (passing through > 600 nm). After observation, the gels were stained with Coomassie brilliant blue R-250 (CBB).

**UV-Vis spectroscopic analysis**

A UV-2600 spectrophotometer (SHIMADZU) was used to monitor the ultraviolet and visible absorption spectra of the proteins. An Opto-Spectrum Generator (Hamamatsu Photonics) was used to prepare monochromatic light of various wavelengths to induce photoconversion: teal-absorbing form, 470–490 nm; green-absorbing form, 490–580 nm; yellow-absorbing form, 580–620 nm; orange-absorbing form, 620–640 nm. Among the AM1_6305g1 and the AM1_1499g1 variants, the molecules containing both green or yellow/teal and orange/green photoconvertible components were irradiated with each monochromatic light independently for conversion into the dark state (15Z–isomer) and the photoproduct state (15E–isomer). The absorption spectra of these native proteins were recorded at r.t., except for the AM1_1499g1 wild-type and R131Q variants, which were measured at low (5°C) and high (30°C) temperatures. Normalized difference absorption spectra (“dark state–photoproduct state” of either photoconvertible components) were calculated from these raw absorption spectra (shown in Fig. S5). Furthermore, the normalized double-difference spectrum (normalized difference spectrum of the yellow/teal component–normalized difference spectrum of the orange/green component) of AM1_1499g1_T159N was calculated from these normalized difference absorption spectra. Photocycle forms were decided by comparing these spectra between each variant molecule and known orange/green, yellow/teal, and green/teal photoconvertible molecules (AM1_1499g1 wild-type, S118C and S118C/Y151L/T159N, respectively)[24].
Identification of the chromophore incorporated into the AM1_6305g1 and AM1_1499g1 variant molecules containing two photoconvertible components

We established the experimental protocol in this study to determine the binding chromophore species of the molecules containing both green-to-yellow/teal and orange/green photoconvertible components. We first prepared the sample in which one photoconvertible component was fixed in the photoproduct state (15E-isomer), while another photoconvertible component was fixed to the dark state (15Z-isomer). The sample under such a condition was subjected to denaturation with 8 M acidic urea (pH < 2.0). Irradiation of the sample with white light (3 min) caused photoconversion of the 15E-isomer of one component. We determined the binding chromophore species of each photoconvertible component based on comparison of the difference spectrum after and before white light illumination (shown in Fig. S6) with those of the PCB-binding and PVB-binding reference molecules (AM1_1499g1 and AM1_6305g1, respectively)[15,24]. Furthermore, the normalized double-difference spectra (normalized difference spectrum of the yellow/teal component–normalized difference spectrum of the orange/green component) of AM1_6305g1_C99S/L132Y/N140T and C99S/Q112R/L132Y/N140T were calculated from these normalized difference absorption spectra.

Data Availability Statement

In this study, we did not obtain any sequence and structural data to deposit in community-approved public repositories. We used sequence and structural information of cyanobacteriochromes (CBCRs) already deposited in the public repositories, such as National Center for Biotechnology Information (NCBI) and Protein Data Bank (PDB), for in silico analysis.
Acknowledgments

We thank Prof. J. Clark Lagarias and Dr. Nathan C. Rockwell (University of California, Davis) for helpful discussion, and Enago (https://www.enago.jp/) for the English language review. This work was supported by Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (JPMJCR1653, to R.N.).

Competing interests

The authors declare no competing interest.
References

1. Fushimi, K. and Narikawa, R. (2019) Cyanobacteriochromes: photoreceptors covering the entire UV-to-visible spectrum. Curr. Opin. Struct. Biol. 57, 39–46.

2. Narikawa, R., Fukushima, Y., Ishizuka, T., Itoh, S. and Ikeuchi, M. (2008) A novel photoactive GAF domain of cyanobacteriochrome AnPixJ that shows reversible green/red photoconversion. J. Mol. Biol. 380, 844–855.

3. Hirose, Y., Shimada, T., Narikawa, R., Katayama, M. and Ikeuchi, M. (2008) Cyanobacteriochrome CcaS is the green light receptor that induces the expression of phycobilisome linker protein. Proc. Natl. Acad. Sci. U. S. A. 105, 9528–9533.

4. Ishizuka, T., Narikawa, R., Kohchi, T., Katayama, M. and Ikeuchi, M. (2007) Cyanobacteriochrome TePixJ of Thermosynechococcus elongatus harbors phycoviolobilin as a chromophore. Plant Cell Physiol. 48, 1385–1390.

5. Moreno, M. V., Rockwell, N. C., Mora, M., Fisher, A. J. and Lagarias, J. C. (2020) A far-red cyanobacteriochrome lineage specific for verdins. Proc. Natl. Acad. Sci. U. S. A. 117, 27962–27970.

6. Narikawa, R., Nakajima, T., Aono, Y., Fushimi, K., Enomoto, G., Ni-Ni-Win, Itoh, S., Sato, M. and Ikeuchi, M. (2015) A biliverdin-binding cyanobacteriochrome from the chlorophyll d-bearing cyanobacterium Acaryochloris marina. Sci. Rep. 5, 7950.

7. Miyake, K., Fushimi, K., Kashimoto, T., Maeda, K., Ni-Ni-Win, Kimura, H., Sugishima, M., Ikeuchi, M. and Narikawa, R. (2020) Functional diversification of two bilin reductases for light perception and harvesting in unique cyanobacterium Acaryochloris marina MBIC 11017. FEBS J.

8. Yoshihara, S., Katayama, M., Geng, X. and Ikeuchi, M. (2004) Cyanobacterial phytochrome-like PixJ1 holoprotein shows novel reversible photoconversion between blue- and green-absorbing forms. Plant Cell Physiol. 45, 1729–1737.

9. Ishizuka, T., Shimada, T., Okajima, K., Yoshihara, S., Ochiai, Y., Katayama, M. and Ikeuchi, M. (2006) Characterization of cyanobacteriochrome TePixJ from a thermophilic cyanobacterium Thermosynechococcus elongatus strain BP-1. Plant Cell Physiol. 47, 1251–1261.

10. Rockwell, N. C., Njuguna, S. L., Roberts, L., Castillo, E., Parson, V. L., Dwojak, S., Lagarias, J. C. and Spiller, S. C. (2008) A second conserved GAF domain cysteine is required for the blue/green photoreversibility of cyanobacteriochrome Thr0924 from Thermosynechococcus elongatus. Biochemistry 47, 7304–7316.

11. Ma, Q., Hua, H.-H., Chen, Y., Liu, B.-B., Krämer, A. L., Scheer, H., Zhao, K.-H. and Zhou, M. (2012) A rising tide of blue-absorbing biliprotein photoreceptors: characterization of seven such bilin-binding GAF domains in Nostoc sp. PCC7120. FEBS J. 279, 4095–4108.

12. Rockwell, N. C., Martin, S. S. and Lagarias, J. C. (2012) Mechanistic insight into the photosensory versatility of DXCF cyanobacteriochromes. Biochemistry 51, 3576–3585.

13. Rockwell, N. C., Martin, S. S., Gulevich, A. G. and Lagarias, J. C. (2012) Phycoviolobilin formation and spectral tuning in the DXCF cyanobacteriochrome subfamily. Biochemistry 51, 1449–1463.

14. Cho, S. M., Jeoung, S. C., Song, J.-Y., Kupriyanova, E. V., Pronina, N. A., Lee, B.-W., Jo, S.-W., Park, B.-S., Choi, S.-B., Song, J.-J., et al. (2015) Genomic survey and biochemical analysis of recombinant candidate cyanobacteriochromes reveals enrichment for near UV/violet sensors in the halotolerant and alkaliphilic cyanobacterium Microcoleus IPPAS B353. J. Biol. Chem. 290, 28502–28514.

15. Hasegawa, M., Fushimi, K., Miyake, K., Nakajima, T., Oikawa, Y., Enomoto, G., Sato,
M., Ikeuchi, M. and Narikawa, R. (2018) Molecular characterization of DXCF cyanobacteriochromes from the cyanobacterium Acaryochloris marina identifies a blue-light power sensor. J. Biol. Chem. 293, 1713–1727.

Narikawa, R., Suzuki, F., Yoshihara, S., Higashi, S.-I., Watanabe, M. and Ikeuchi, M. (2011) Novel photosensory two-component system (PixA-NixB-NixC) involved in the regulation of positive and negative phototaxis of cyanobacterium Synechocystis sp. PCC 6803. Plant Cell Physiol. 52, 2214–2224.

Song, J.-Y., Cho, H.-S., Cho, J.-I., Jeon, J. C. and Park, Y.-I. (2011) Near-UV cyanobacteriochrome signaling system elicits negative phototaxis in the cyanobacterium Synechocystis sp. PCC 6803. Proc. Natl. Acad. Sci. U. S. A. 108, 10780–10785.

Narikawa, R., Kohchi, T. and Ikeuchi, M. (2008) Characterization of the photoactive GAF domain of the CikA homolog (SyCikA, Slr1969) of the cyanobacterium Synechocystis sp. PCC 6803. Photochem. Photobiol. Sci. 7, 1253–1259.

Enomoto, G., Hirose, Y., Narikawa, R. and Ikeuchi, M. (2012) Thiol-based photocycle of the blue and teal light-sensing cyanobacteriochrome Tlr1999. Biochemistry 51, 3050–3058.

Ishizuka, T., Kamiya, A., Suzuki, H., Narikawa, R., Noguchi, T., Kohchi, T., Inomata, K. and Ikeuchi, M. (2011) The cyanobacteriochrome, TePixJ, isomerizes its own chromophore by converting phycocyanobilin to phycoviolobilin. Biochemistry 50, 953–961.

Burgie, E. S., Walker, J. M., Phillips, G. N., Jr and Vierstra, R. D. (2013) A photo-labile thioether linkage to phycoviolobilin provides the foundation for the blue/green photocycles in DXCF-cyanobacteriochromes. Structure 21, 88–97.

Cornilescu, C. C., Cornilescu, G., Burgie, E. S., Markley, J. L., Ulijasz, A. T. and Vierstra, R. D. (2014) Dynamic structural changes underpin photoconversion of a blue/green cyanobacteriochrome between its dark and photoactivated states. J. Biol. Chem. 50, 1870–1874.

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C. and Ferrin, T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612.
Figure Legends

Figure 1. Absorption spectra of native wild-type and variant molecules of the AM1_6305g1 scaffold.

(A–D) Absorption spectra of the dark state (solid gray line) and the photoproduct state (solid orange line) were measured at room temperature. In the case of molecules containing two photoconvertible components, both components were fixed to the dark state or the photoproduct state. Wavelength area corresponding to the positive (dark state, deep color triangle) and negative (photoproduct state, light color triangle) peaks of the normalized difference spectra from the two photoconvertible components (yellow/teal component in short wavelength region, cyan; orange/green component in the long-wavelength region, magenta) were assigned by colored triangles. The normalized difference spectra are shown in Figure 2. (A) The molecules having Gln, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, respectively. (B) The molecules having Gln, Tyr, and Thr in these positions. (C) The molecules having Arg, Leu, and Asn in these positions. (D) The molecules having Arg, Tyr, and Thr in these positions. (i, ii) The molecules having Cys (i) or Ser (ii) in the second Cys position.

Figure 2. Normalized difference absorption spectra (dark state–photoproduct state) of native wild-type and variant molecules of the AM1_6305g1 scaffold.

(A–D) Difference spectra of the molecules containing a single photoconvertible component (solid gray line) were calculated from the native absorption spectra shown in Figure 1. On the other hand, difference spectra of the molecules containing two photoconvertible components (yellow/teal component in the short wavelength region, solid cyan line; orange/green component in the long-wavelength region, solid magenta line) were calculated from the native absorption spectra shown in Figure S5. In Figure S5, we separately excited each
photoconvertible component. These spectra were compared with those of the known orange/green, yellow/teal, and green/teal photoconvertible molecules (AM1_1499g1 wild-type, orange dotted line; S118C/Y151L/T159N, green dotted line, respectively). These absorption peaks are summarized in Table 1. (A) The molecules having Gln, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, respectively. (B) The molecules having Gln, Tyr, and Thr in these positions. (C) The molecules having Arg, Leu, and Asn in these positions. (D) The molecules having Arg, Tyr, and Thr in these positions. (i, ii) The molecules having Cys (i) or Ser (ii) in the second Cys position.

Figure 3. Normalized difference absorption spectra (dark state–photoproduct state) of denatured AM1_6305g1 species.

(A–D) Difference spectra of the molecules containing a single photoconvertible component (solid gray line) and two photoconvertible components (yellow/teal component in the short wavelength region, solid cyan line; orange/green component in the long-wavelength region, solid magenta line) were calculated from the denatured absorption spectra shown in Figure S6. Detailed experimental protocol was described in Materials and Methods. Furthermore, the normalized double-difference spectra (yellow/teal photoconvertible component in the short wavelength region, solid violet line) of AM1_6305g1_C99S/L132Y/N140T and C99S/Q112R/L132Y/N140T were calculated from these normalized difference absorption spectra. These spectra were compared with those of the known PCB- and PVB-binding molecules (AM1_1499g1 wild-type, orange dotted line; AM1_6305g1 wild-type, green dotted line, respectively). These absorption peaks are summarized in Table 1. (A) The molecules having Gln, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, respectively. (B) The molecules having Gln, Tyr, and Thr in these positions. (C) The molecules having Arg, Leu,
and Asn in the positions. (D) The molecules having Arg, Tyr, and Thr in these positions. (i, ii) The molecules having Cys (i) or Ser (ii) in the second Cys position.
Figure 4. UV-Vis spectra of native and denatured AM1_6305g1_C99A.

(A) The absorption spectra (dark state, solid gray line; photoproduct state, solid yellow line) of AM1_6305g1_C99A were measured at room temperature. Wavelength area of the positive (dark state, deep color triangle) and negative (photoproduct state, light color triangle) peaks of the normalized difference spectra from the two photoconvertible components (green/teal component in short wavelength region, cyan; orange/green component in the long-wavelength region, magenta) were assigned by colored triangles. (B, C) The normalized difference spectra (dark state–photoproduct state) of the molecules containing the two photoconvertible components (green/teal component in the short wavelength region, cyan solid line; orange/green component in the long-wavelength region, solid magenta line) were calculated from the native (B) and denatured (C) absorption spectra shown in Figure S5 and Figure S6. The native spectra were compared with those of the known orange/green, yellow/teal, and green/teal photoconvertible molecules (AM1_1499g1 wild-type, orange dotted line; S_{118}C, yellow dotted line; S_{118}C/Y_{151}L/T_{159}N, green dotted line, respectively). On the other hand, the denatured spectra were compared with those of the known PCB- and PVB-binding molecules (AM1_1499g1 wild-type, orange dotted line; AM1_6305g1 wild-type, green dotted line, respectively). These absorption peaks are summarized in Table 1.

Figure 5. Absorption spectra of native AM1_1499g1 species.

(A–D) Absorption spectra of the dark state (solid gray line) and the photoproduct state (solid orange line) were measured at room temperature, except for the wild-type and R_{131}Q variant molecules measured at low temperature. In the case of molecules containing two photoconvertible components, both components were fixed to the dark state or the photoproduct state. Wavelength area of the positive (dark state, deep color triangle) and
negative (photoproduct state, light color triangle) peaks of the normalized difference spectra from the two photoconvertible components (green/teal one in short wavelength region, cyan; orange/green one in the long-wavelength region, magenta) were assigned by colored triangles. The normalized difference spectra are shown in Figure 6. (A) The molecules having Gln, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, respectively. (B) The molecules having Gln, Tyr, and Thr in these positions. (C) The molecules having Arg, Leu, and Asn in these positions. (D) The molecules having Arg, Tyr, and Thr in these positions. (i, ii) The molecules having Cys (i) or Ser (ii) in the second Cys position.

Figure 6. Normalized difference absorption spectra (dark state–photoproduct state) of native AM1_1499g1 species.

(A–D) Difference spectra of the molecules containing a single photoconvertible component (solid gray line) were calculated from the native absorption spectra shown in Figure 5. On the other hand, difference spectra of the molecules containing two photoconvertible components (green/teal component in short wavelength region, solid cyan line; orange/green component in the long-wavelength region, solid magenta line) were calculated from the native absorption spectra shown in Figure S5. In Figure S5, we separately excited each photoconvertible component. These spectra were compared with those of known orange/green, yellow/teal, and green/teal photoconvertible molecules (AM1_1499g1 wild-type, orange dotted line; S_{118}C, yellow dotted line; S_{118}C/Y_{131}L/T_{159}N, green dotted line, respectively). These absorption peaks are summarized in Table 1. (A) The molecules having Gln, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, respectively. (B) The molecules having Gln, Tyr, and Thr in these positions. (C) The molecules having Arg, Leu, and Asn in these positions. (D) The molecules having Arg, Tyr, and Thr in these positions. (i, ii) The molecules having Cys (i) or Ser (ii) in the second Cys position.
Figure 7. Normalized difference absorption spectra (dark state–photoproduct state) of denatured AM1_6305g1 species.

(A–D) Difference spectra of the molecules containing single photoconvertible component (solid gray line) and two photoconvertible components (green/teal component in the short wavelength region, solid cyan line; orange/green component in the long-wavelength region, solid magenta line) were calculated from the denatured absorption spectra shown in Figure S6. Detailed experimental protocol was described in the Materials and Methods. These spectra were compared with those of known PCB- and PVB-binding molecules (AM1_1499g1 wild-type, orange dotted line; AM1_6305g1 wild-type, green dotted line, respectively). These absorption peaks were summarized in Table 1. (A) The molecules having Gln, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, respectively. (B) The molecules having Gln, Tyr, and Thr in these positions. (C) The molecules having Arg, Leu, and Asn in these positions. (D) The molecules having Arg, Tyr, and Thr in these positions. (i, ii) The molecules having Cys (i) or Ser (ii) in the second Cys position.

Figure 8. Molecular evolution of DXCF CBCR GAF domains in AM1_1499g1/AM1_6305g1 lineage.

(A) Possible molecular mechanisms for PCB-to-PVB isomerization. (i) PCB incorporation into apo DXCF CBCR GAF domain by self-ligation activity. The incorporated chromophore forms a covalent bond between its C3¹ position and the first Cys of the apo protein. (ii) Interaction network after PCB incorporation in the TePixJg scaffold (typical blue/green photoconvertible molecule which has Arg, Leu, and Asn in the Gln/Arg, Leu/Tyr, and Asn/Thr positions, with the second Cys). The bent PCB is dually fixed at the A-ring and C10 position by the first and second Cys residues with the ligation. This dual fixation mode results
in distortion of the C5 bridge between A- and B-rings and promotes PCB-to-PVB isomerization. (iii) Interaction network after PCB incorporation in the AM1_6305g1 scaffold having the second Cys (green/teal photoconvertible molecule which has Gln, Leu, and Asn in these positions, with the second Cys). The unbent PCB is dually fixed at the A-ring and C10 bridge by the first and second Cys residues, although the second Cys does not form covalent bonds. (iv) Interaction network after PCB incorporation in the AM1_6305g1 scaffold lacking the second Cys (C99S variant molecule which has Gln, Leu, and Asn in these positions, without the second Cys). The unbent PCB is fixed at the A-ring by the first Cys residue. At the other side, the C10 bridge of the chromophore is weakly held by the Ser residue instead of the second Cys residue, which is supported by the three key residues near the ring D. (v) Interaction network after PCB incorporation in the AM1_1499g1 scaffold (orange/green photoconvertible molecule which has Arg, Tyr, and Thr in these positions, without the second Cys). Because of the lack of neither the second Cys nor the three key residues, no PCB-to-PVB isomerization activity is observed without distortion at the C5 bridge. (B) Phylogenetic tree of diversified CBCR GAF domains with phytochrome GAF domains as the outgroup, which was constructed based on the alignment shown in Figure S7. (i) Each lineage cluster in the whole tree is classified roughly according to photocycle (AM1_1499g1/AM1_6305g1, Asp-Xaa-Cys-Phe (DXCF), expanded red/green (XRG), and the other lineages). CBCR subfamilies possessing the DXCF motif were indicated with cyan. (ii) The AM1_1499g1/AM1_6305g1 lineage is enlarged from the whole tree. Orange, green, and blue lines show the possible emergence and trace of the orange/green, green/teal, and blue/teal CBCR GAF domains, respectively.
Table 1. Wavelength peaks of the difference absorption spectra of the AM1_6305g1 and AM1_1499g1 variant molecules under native and denatured conditions (shown in Figures 2, 3, 4, 6, and 7).

| AM1_6305g1 | Native | Denatured | AM1_1499g1 | Native | Denatured | Bilin | Mutation site |
|------------|--------|-----------|------------|--------|-----------|------|---------------|
|            | Dark  – Photoproduct | Dark  – Photoproduct |            | Dark  – Photoproduct | Dark  – Photoproduct |      |               |
|            | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Bilin | Mutation site |
|            | (nm)     | (nm)      | (nm)     | (nm)      | (nm)     | (nm)      | (nm)     | (nm)      | (nm)     | (nm)      |      |               |
| Wild-type  |          |          |          |          |          |          |          |          |          |          |      |               |
| S114C/R133Q/Y151L/T159N | 558 | 492 | 601 | 508 |          |          |          |          |          |          |      |               |
| L132Y/N140T |          |          |          |          |          |          |          |          |          |          |      |               |
| S114C/R133Q | 576 | 491 | 601 | 508 |          |          |          |          |          |          |      |               |
| Q112R      |          |          |          |          |          |          |          |          |          |          |      |               |
| S114C/Y151L/T159N | 561 | 492 | 600 | 508 |          |          |          |          |          |          |      |               |
| C96S       |          |          |          |          |          |          |          |          |          |          |      |               |
| R131Q/Y151L/T159N | 557 | 491 | 601 | 509 |          |          |          |          |          |          |      |               |
| C96S/L132Y/N140T |          |          |          |          |          |          |          |          |          |          |      |               |
| R131Q'     | 575 | 492 | 600 | 512 |          |          |          |          |          |          |      |               |
| C96S/Q112R |          |          |          |          |          |          |          |          |          |          |      |               |
| Y151L/T159N | 561 | 492 | 601 | 508 |          |          |          |          |          |          |      |               |
| C96S/Q112R/L132Y/N140T |          |          |          |          |          |          |          |          |          |          |      |               |
| Wild-type | 577 | 491 | 593 | 507 |          |          |          |          |          |          |      |               |
| C96S      |          |          |          |          |          |          |          |          |          |          |      |               |
| PVB       |          |          |          |          |          |          |          |          |          |          |      |               |
| Cys / Ser |          |          |          |          |          |          |          |          |          |          |      | Leu / Tyr / Asn / Thr |
| Gln / Arg |          |          |          |          |          |          |          |          |          |          |      |               |
| Leu / Tyr |          |          |          |          |          |          |          |          |          |          |      |               |
| Asn / Thr |          |          |          |          |          |          |          |          |          |          |      |               |
| a. These values were calculated from double-difference spectra (normalized difference spectrum yellow/orange/green component).
| b. Their photochemical properties were recorded under low temperature (5°C).
**Figure 1.**

**A.** UV–visible absorption spectra of C99A variants of AM1_6305g1 (Cys/Ala) in both native and denatured states. The spectra show changes in absorbance at different wavelengths, indicating photostability differences between variants.

**B.** Normalized difference in absorbance (ΔABS) for Gln/Arg, Leu/Tyr, and Asn/Thr variants in native and denatured states. The data illustrates the photostability and structural changes under different conditions.

**C.** Similar to **B**, but for Cys/Ala variants, highlighting the differences in photostability and structural changes between native and denatured states.

**Legend:**
- Dark
- Photo
- Dark – Photo
- Native
- Denatured
- Po – Pg
- Py – Pt
- Pg – Pt
- PCB
- PVB
