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Minireview

Biosynthesis of chromophores for phytochrome and related photoreceptors

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Abstract A broad group of open tetrapyrroles derived from heme are collectively called bilins. Phycocyanobilin and phycoerythrobilin are utilized as accessory chromophore for light harvesting by phycobiliproteins that comprise the photosynthetic apparatus in cyanobacteria and algae. Phytochromes, known as a major photoreceptor in plants, contain a phytochromobilin chromophore as an essential prosthetic group for photo-sensing. The possible ancestral proteins of phytochromes, as found in cyanobacteria and eubacteria, possess phycocyanobilin and/or biliverdin as a prosthetic group. In this mini-review, the diverse functions of bilin chromophores and their biosynthesis in photosynthetic organisms are summarized and the potential applications to plant biotechnology will be discussed.

Key words: Ferredoxin-dependent bilin reductase, heme oxygenase, photoreceptor, phytochrome chromophore, tetrapyrrole biosynthesis.

Tetrapyrroles, which are also referred to as porphyrins, are important molecules in living cells (Smith and Witty 2002). The major tetrapyrroles in nature are heme, chlorophylls, and bilins (Figure 1). Heme and chlorophylls tightly bind iron and magnesium, respectively, while linear tetrapyrrole bilins are free of metals. Heme, which often forms the prosthetic group of metalloenzymes, is essential in various biological reactions of oxidation and reduction. Chlorophylls, the most abundant tetrapyrroles on Earth, are utilized as accessory and antenna molecules in the photosystems of the photosynthetic machinery. Bilins as well as chlorophylls are key components for photoperception in photosynthetic organisms, because they contain rings rich in conjugated double bonds that absorb light effectively. Substitution of side chains on the ring structure of different double bonds in tetrapyrroles alters the specificity of light absorption. Therefore the physical properties of tetrapyrroles are suitable for photo-sensing systems. In this mini-review, we summarize the biosynthesis and potential application of bilins in photosynthetic organisms.

Phytochromes and phytochrome-related proteins

Photosynthetic organisms possess sophisticated photosensory systems to adapt their growth and development to light conditions that are comprised of wavelength, fluence, direction, and duration of illumination. According to photochemistry laws, light must firstly be absorbed by certain chemicals in order to elicit an action, and this is unequivocally true in living systems. Prosthetic groups attached to the photoreceptor are essential for photo-sensing and are called the chromophore. Phytochromes are a family of photoreceptors used to sense mainly red light (R) and far-red light (FR), although type I phytochrome that accumulates to very high levels in imbibed seeds is a very sensitive sensor of almost all wavelengths of light in order to trigger for germination (Furuya and Schäfer 1996). In vascular plants, phytochromes have phytochromobilin (PΦB) as their chromophore. Although phytochromes forms a small multiple gene family (e.g. five phytochromes phyA to phyE in *Arabidopsis thaliana* and three in rice; Clack et al. 1994), PΦB is thought to be the common chromophore in all phytochromes in vascular plants (Rüdiger and Thümmler 1992).

Phytochromes that were first described in late 1950's had supposedly been unique to land plants. However, phytochrome-related genes were identified in cyanobacteria by the genome sequencing project (Kaneko et al. 1996). Structures of phytochrome and

Abbreviations: BV IXα, biliverdin IXα; FR, far-red light; PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; Proto IX, proto-porphyrin IX; R, red light.

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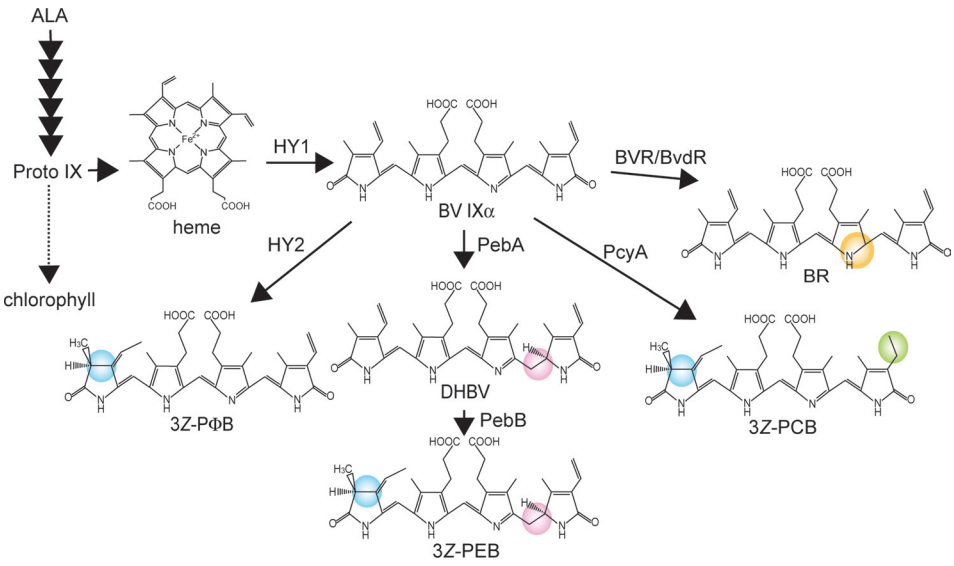

Figure 1. Chemical structures and biosynthetic pathways of chlorophyll, heme and bilins. BV is synthesized from 5-aminolevulinic acid via heme. BV is further reduced to produce PΦB in plants, PEB and PCB in algae and BR in animals by the action of different bilin reductase. Note that no obvious sequence similarity was detected between BV reductases (BVR and bvdR) and other ferredoxin-dependent bilin reductases (HY2, pcyA, pebA and pebB). See text for gene symbols and abbreviations.

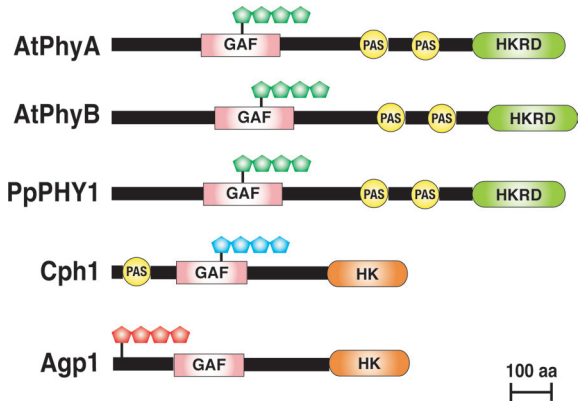

Figure 2. Comparison of domain structures among phytochrome and phytochrome-related proteins. Domain structures of phytochrome and phytochrome-related proteins are schematically illustrated. AtPhyA, AtPhyB in *Arabidopsis*, PpPHY1 in *Physcomitrella patens*, Cph1 in *Synechocystis* PCC6803 and Agp1 in *Agrobacterium* are shown. Chromophore molecules PΦB, PCB, and BV are shown in green, blue, and red, respectively.

related proteins are schematically shown in Figure 2. The *Synechocystis* PCC6803 genome encodes several phytochrome-related proteins. One of the proteins, Cph1, showed histidine kinase activity although its biological function is still unknown (Yeh et al. 1997). The Cph1 protein expressed in *E. coli* could ligate exogenous phycocyanobilin (PCB), and showed photoreversible spectral properties. This indicates that the cyanobacterial proteins are ancestral to or at least related to phytochrome (Yeh et al. 1997). Both plant and cyanobacterial phytochrome have a chromophore-binding domain in the N-terminal half. Although certain homology in the C-terminal half was observed between phytochromes and phytochrome-related proteins, cyanobacterial phytochrome-related proteins have typical histidine kinase motifs that were disrupted in land plants. Another difference in these two families was in chromophore structure. The Cph1 protein was purified from cyanobacteria that expressed His-tagged Cph1 protein and judging from its spectral characteristics the chromophore was PCB (Hübschmann et al. 2001).

Furthermore, phytochrome-related proteins were found in eubacteria and named bacteriophytochromes, including Agp1 in *Agrobacterium tumefaciens*, where the chromophore was found to be biliverdin (BV) (Bhoo et al. 2001). Phytochrome would most likely be derived from ancestral molecules that served as sensors of bilins and light (Montgomery and Lagarias 2002). Bacteriophytochromes are expected to be an ancestral form of plant phytochrome from an evolutional point of view. However, the chromophore binding sites of these proteins differ. The binding site of BV in Agp1 is the cysteine residue close to N-terminus (Cys-20) while plant phytochromes and some cyanobacterial phytochromes have the chromophore bound to the GAF domain (Lamparter et al. 2002). Phylogenetic analysis suggests that the protein evolution and selection of chromophore-binding sites occurred independently (Lamparter 2004). What is the evolutionary consequence of having related but different chromophores in those photoreceptors? One of the effective approaches to answer this question is the utilization of chromophore substitution systems where knowledge of chromophore biosynthesis is applied in vivo, as described later.

Bilins and their biosynthesis in photosynthetic organisms

Bilins are a collective common name for linear tetrapyrroles. The major bilins in nature are BV, bilirubin (BR), PCB, phycoerythrobilin (PEB) and PΦB, and they are all derived from heme. The structures are summarized in Figure 1. Heme and chlorophyll share a common biosynthetic pathway from aminolevulinic acid to protoporphyrin (Proto) IX. Heme, which contains iron, is synthesized from Proto IX by ferrochelatase, while chlorophyll, with magnesium, is synthesized by Mg-chelatase. Heme is an essential prosthetic group in many reduction/oxidation processes. Heme is cleaved to BV IXα by heme oxygenase which is a degradation process in animals but for photosynthetic organisms this step is a biosynthetic process (Muramoto et al. 1999). Further reduced bilins are synthesized from BV IXα by the action of different bilin reductases (Kohchi et al. 2001; Frankenberg et al. 2001). In photosynthetic eukaryotes the full set of enzymes for bilin biosynthesis are localized to the plastid compartment.

Heme Oxygenase

Heme oxygenase catalyzes the stereospecific cleavage of protoheme to BV IXα by three successive monooxygenation steps and the release of both carbon monoxide and ferric iron. The common role of heme oxygenase is the degradation of heme, but there are a wide variety of additional roles in nature including iron acquisition, carbon monoxide signaling, and bilin biosynthesis. In plants, a gene for heme oxygenase was first identified from the *Arabidopsis* photomorphogenetic mutant *hy1* (Davis et al. 1999; Muramoto et al. 1999). The *hy1* and *hy2* mutants recognized by hypocotyl elongation when grown in light do not show response to R and FR although phytochrome protein synthesis is normal (Chory et al. 1989). *HY1* encoded active heme oxygenase for phytochrome chromophore biosynthesis (Muramoto et al. 1999). Thereafter several equivalent photomorphogenetic mutants in different species have been identified; *se5* in rice, *yg-2* in tomato, *pcd1* in pea and *ptr116* in moss (summarized in Terry et al. 2002). These mutants are useful for functional studies of phytochromes in each species. In addition to their respective biological functions, the enzymatic characters of heme oxygenases from plants are substantially different from animal ones. Plant heme oxygenases are ferredoxin-dependent enzymes localized to the plastid compartment, while animal heme oxygenase is a microsomal enzyme coupled to NADPH-dependent P450 reductase. Iron release in the plant heme oxygenase reaction seemed to be tightly regulated *in vivo* since the presence of iron chelator that is not required for animal and bacterial enzymes is essential for the *in vitro* reaction (Muramoto et al. 2002).

Heme oxygenase in *Arabidopsis* is encoded by a small gene family of four genes, *AtHO1* (*HY1*) to *AtHO4* (Davis et al. 2001, Terry et al. 2002). Three gene products (AtHO2 to AtHO4) originally identified by sequence similarity to AtHO1 showed enzymatic activity *in vitro* using *E. coli* and yeast expressed proteins and are potentially localized in plastids (PJL, unpublished results). However, the temporal and spatial expression profiles of these heme oxygenases slightly overlapped with that of *HY1* and their expression levels are much lower than that of *HY1*. This difference is probably the reason why the *hy1* mutant was isolated in spite of genetic redundancy, and individual heme oxygenases may have specific roles in different developmental stages and events.

Ferredoxin-dependent bilin reductases

The metabolism of BV is different in animals, algae, and plants. Although it is a common feature that further reduction reactions occur in all organisms, the double-bond specificities of these reactions are unique to different organisms (Figure 1). The BV reductase first identified in mammals catalyzes a two-electron reduction at the double bond between ring B and ring C of BV to produce BR (10,11-reductase). A similar enzyme, encoded by *bvdR*, was found in cyanobacteria but its biological significance remains to be solved (Schluchter and Glazer 1997).

In plants BV is a precursor of PΦB, the chromophore molecule of phytochromes. The *Arabidopsis hy2* mutant, which is defective in phytochrome responses, was a good candidate gene for phytochrome chromophore biosynthesis. The *HY2* gene was isolated by map-based cloning (Kohchi et al. 2001). The deduced protein showed no sequence similarity to functionally identified proteins and was a soluble protein that contained a transit peptide for plastid import at its N-terminal region. The protein expressed in *E. coli* exhibited ferredoxin-dependent PΦB synthase activity *in vitro*, which is a two-electron reduction at the double-bond at C2 (2, 3-reductase). It was confirmed that the reaction product was PΦB, chemically by HPLC and biophysically by generation of photoactive phytochrome through mixing the reaction products with phytochrome apoprotein. The plastid localization of HY2 was confirmed experimentally, and therefore the entire biosynthesis of PΦB is localized to the plastid compartment (Kohchi et al. 2001).

In cyanobacteria and algae (rhodophytes and cryptomonads) linear tetrapyrrole precursors PCB and PEB are predominantly used in their light-harvesting phycobiliprotein complexes. Extensive studies with red

algae suggested that these tetrapyrroles are synthesized from BV by specific bilin reductases, but molecular analysis was difficult mainly due to the low expression levels of the bilin reductase. By similarity searches with the amino acid sequence of PΦB synthase encoded by *HY2*, genes encoding putative bilin reductases were discovered in the genomes of microorganisms that contain phycobilins (Frankenberg et al. 2001). By the enzymatic analysis of the expressed proteins, three classes of bilin reductases for phycobilin biosynthesis were identified. They showed ferredoxin-dependent oxidoreductase activities with distinct substrate and product specificities (Figure 1). These novel bilin reductases were involved in PCB and PEB biosynthesis and named PcyA, PebA and PebB (Frankenberg et al. 2001). The PcyA protein is PCB:ferredoxin oxidoreductase ((2, 3), (18^1,18^2)-reductase) for four electron reduction of BV. PebA and PebB are dihydrobiliverdin:ferredoxin oxidoreductase (15,16-reductase) and PEB:ferredoxin oxidoreductase (2,3-reductase), respectively, for two successive reactions from BV to PEB.

Phytochrome chromophore modification by transgenic expression of bilin reductase

The attachment of chromophore is essential for photoreceptors. Plant phytochromes use PΦB as a chromophore, while cyanobacterial phytochromes use PCB. Assembly between chromophore and apophytochrome protein is autocatalytic, and apophytochrome can ligate to various bilins between the A-ring ethylidene group of bilin and the cysteine residue at the chromophore-binding domain *in vitro*. PCB, which is easily obtained from algae, has been used as a substitute compound for PΦB in reconstitution experiments for phytochromes from higher plants. PCB-apophytochrome adducts are structurally photoreversible although they show blue-shifted absorption spectra owing to the reduced number of double-bonds. To evaluate structure requirements and the functional significance of PΦB as the phytochrome chromophore in plants, two experimental systems of chromophore substitution have been reported. The first system was feeding of PCB to a chromophore-deficient mutant (Hanzawa et al. 2001). Due to the technical reasons related to the feeding method the observable phenotypes in this experiment were limited. It is, however, advantageous that various synthetic analogs can be fed to seedlings and be tested in structure-activity relationship studies. The second system was a genetic approach to modify phytochrome chromophore by synthesizing the bilin of interest with plastid-targeted bilin reductase in plants. As shown in Figure 3, the phytochrome-chromophore deficient *hy2* mutant that lacked a gene for

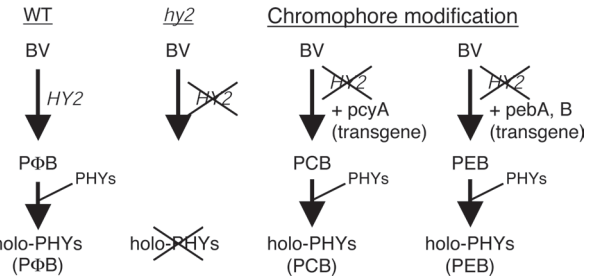

Figure 3. Genetic systems to modify phytochrome chromophore. PΦB synthase is inactivated in the *Arabidopsis hy2* mutant. BV is further reduced to bilins of interest by transgenic expression of ferredoxin-dependent bilin reductases of different specificities and located to the plastid compartment in *hy2*. Phytochrome apoprotein can autocatalytically bind various bilins between the A-ring ethylidene group of bilins and the cysteine residue at the chromophore-binding domain.

PΦB synthase was transformed with the constitutively expressed gene for various bilin reductases. The transformants with pcyA produced PCB and those with both pebA and pebB produced PEB. As a consequence of metabolic engineering of bilins, the phytochrome chromophore was substituted with the biosynthesized bilins (Kami et al. 2004, Muramoto et al. unpublished observation).

Using these systems, the effects of chromophore substitution were analyzed. It was first suspected that the PΦB chromophore was required for the phyA-mediated high irradiance response to FR and was not displaceable with PCB chromophore (Hanzawa et al. 2002). Using a transgenic approach, the wavelength dependency FR high irradiance response mediated by phyA was carefully analyzed. It was clearly shown that the FR high irradiance response was fully functional with PCB chromophore but the effective wavelength was shifted significantly to shorter wavelengths (Kami et al. 2004, Kami et al. unpublished observation). This is the first example of changing the light response by shifting wavelength dependency with chromophore modification of the photoreceptor. In addition, the dark reversion of phytochrome B from R-absorbing form to FR-absorbing form is also affected by the chromophore modification (Kami et al. in preparation).

The genetic modification of chromophore with the genes for bilin reductase should be applicable to any plant species where mutants for PΦB synthase are available. The *aurea* mutants of tomato that have been widely used as phytochrome-deficient mutants, has mutation in a gene for PΦB synthase in tomato (Muramoto et al. 2005). Tomato is more suitable for biochemical analysis than *Arabidopsis* and the modification of phytochrome chromophore in tomato is in progress. We propose that the bilin reductase genes are potential genetic tools for crops to optimize light responses by adjusting wavelength dependency and

sensitivity to light in the conditioned environment.

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