

Protein family review

## The cryptochromes

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### Summary

Cryptochromes are photoreceptors that regulate entrainment by light of the circadian clock in plants and animals. They also act as integral parts of the central circadian oscillator in animal brains and as receptors controlling photomorphogenesis in response to blue or ultraviolet (UV-A) light in plants. Cryptochromes are probably the evolutionary descendents of DNA photolyases, which are light-activated DNA-repair enzymes, and are classified into three groups - plant cryptochromes, animal cryptochromes, and CRY-DASH proteins. Cryptochromes and photolyases have similar three-dimensional structures, characterized by an  $\alpha/\beta$  domain and a helical domain. The structure also includes a chromophore, flavin adenine dinucleotide (FAD). The FAD-access cavity of the helical domain is the catalytic site of photolyases, and it is predicted also to be important in the mechanism of cryptochromes.

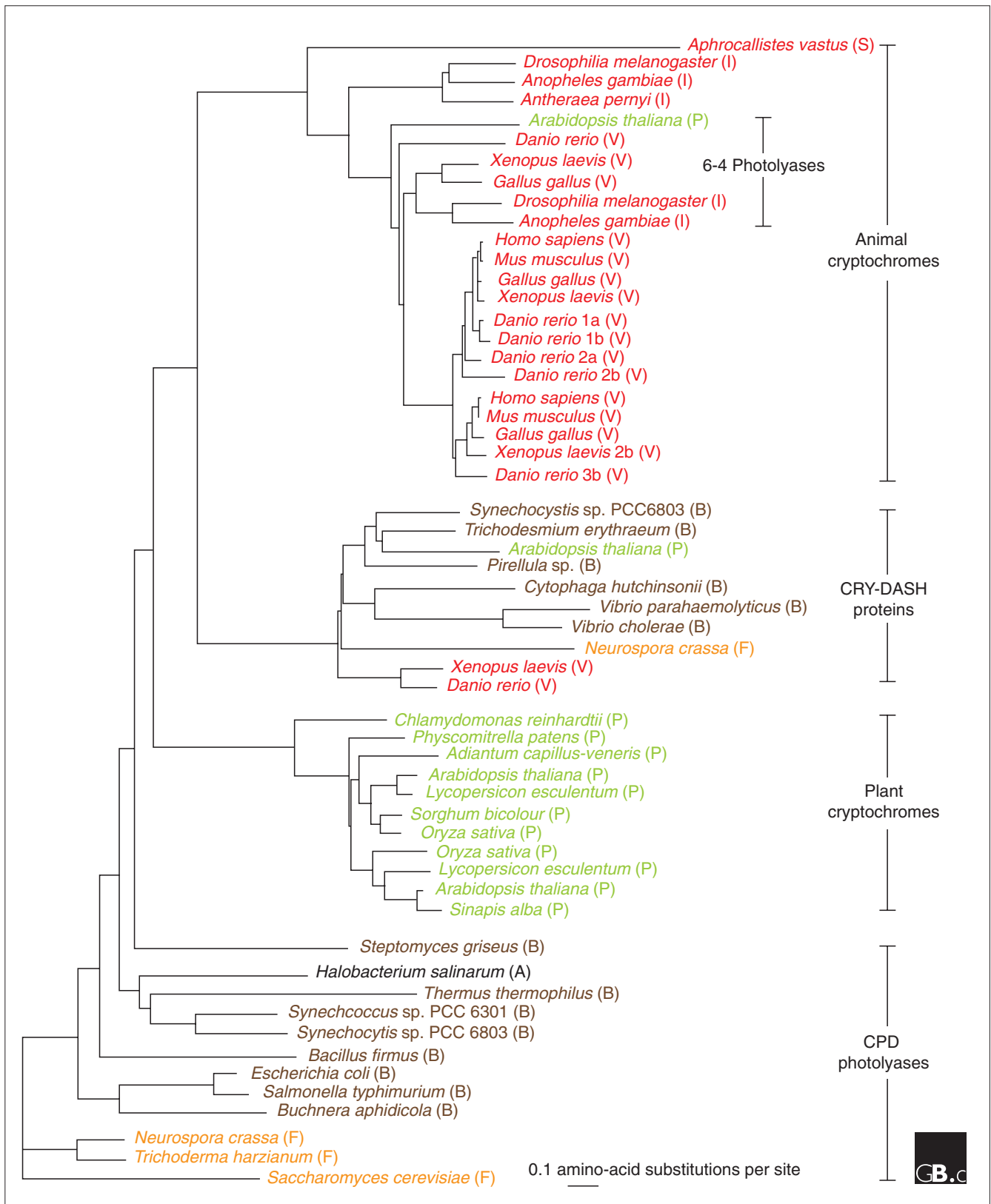
### Gene organization and evolutionary history

Cryptochromes are receptors for blue and ultraviolet (UV-A) light that share sequence similarity to DNA photolyases, DNA-repair enzymes that use blue light to repair UV-induced DNA damage by removing pyrimidine dimers from DNA [1]; cryptochromes have no photolyase activity, however [1-4]. There are two types of DNA photolyase, which repair different types of damage: CPD photolyases repair cyclobutane pyrimidine dimers (CPDs), and 6-4 photolyases repair 6-4 pyrimidine pyrimidone photoproducts. These photolyases together with the cryptochromes make up the photolyase/cryptochrome superfamily [5]. According to their sequence similarities, cryptochromes from a range of organisms can be clustered, more or less, into three subfamilies (Figure 1): plant cryptochromes, animal cryptochromes and cryptochrome-DASH proteins (CRY-DASH; see below).

Cryptochromes are widely distributed in bacteria and eukaryotes but are not found in archaea, although archaea do have a CPD photolyase (see Figure 1). The first cryptochrome gene to be identified was *Arabidopsis CRY1* [6], and cryptochromes

were soon found by homology in other plant species and in animals. Soon after the cloning of the first 6-4 photolyase from *Drosophila* [7,8], a related sequence was discovered in the human expressed sequence tag (EST) databases that proved to encode human cryptochrome 1 (hCRY1) [8,9]. Cryptochromes have now been found in various animal lineages, including insects, fish, amphibians, and mammals. Animal cryptochromes act as components of the circadian clock that control daily physiological and behavioral rhythms and as photoreceptors that mediate entrainment of the circadian clock to light [3].

It was initially thought that only higher eukaryotes had cryptochromes and that prokaryotes had photolyases but not cryptochromes, but further searches of the more recently available genome databases revealed the presence of a cryptochrome gene in cyanobacteria (*Synechocystis*) [10]. This new type of cryptochrome was referred to as CRY-DASH, to underscore its relationship with cryptochromes found in *Drosophila*, *Arabidopsis*, *Synechocystis*, and *Homo* (although CRY-DASH itself is not found in *Drosophila* or



**Figure 1**

An unrooted phylogenetic tree of the photolyase/ cryptochrome superfamily, with subfamilies indicated on the right. Abbreviations: A, archaea; B, bacteria; F, fungi; I, insects; P, plants; S, sponges; V, vertebrates.

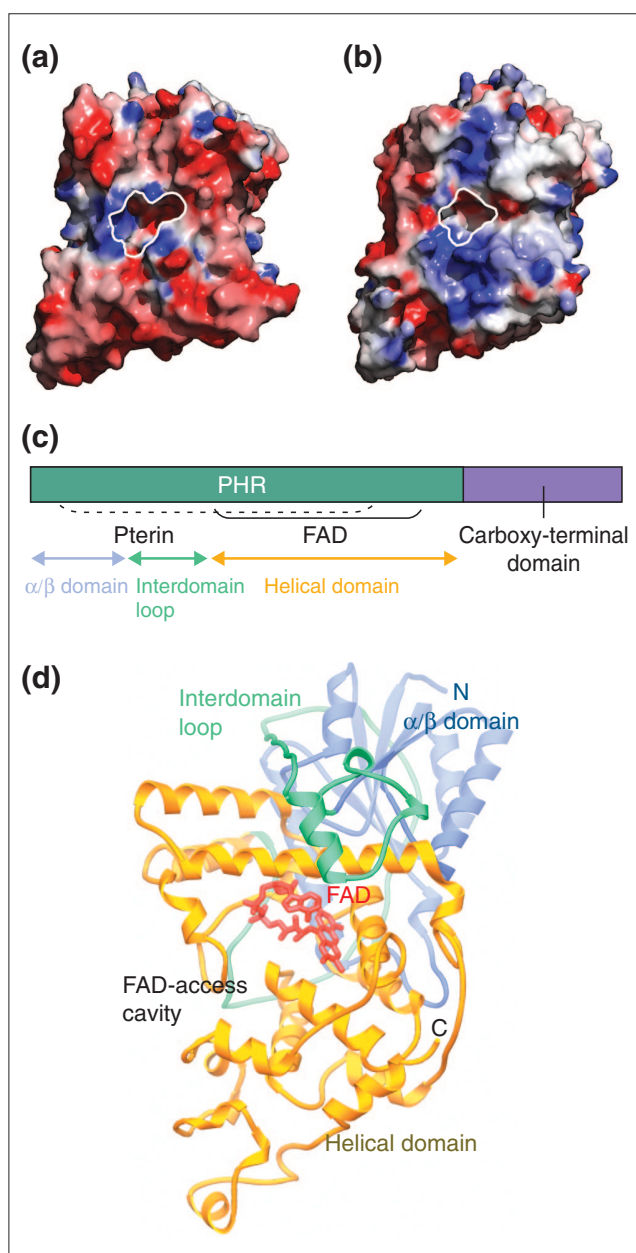
humans) [11]. CRY-DASH proteins have been found not only in the photosynthetic cyanobacteria but also in non-photosynthetic bacteria, fungi, plants and animals, including *Arabidopsis*, *Neurospora*, zebrafish, and *Xenopus* (see Figure 1). The biological function of CRY-DASH proteins remains unknown at present.

The phylogenetic tree shown in Figure 1 illustrates the evolutionary relationships of photolyases and cryptochromes from different organisms. According to the tree topology, the photolyase/cryptochrome superfamily contains four sub-families: animal cryptochromes/6-4 photolyase, plant cryptochromes, CRY-DASH proteins, and CPD photolyases. It is intriguing that animal cryptochromes and 6-4 photolyases are clustered together into the same clade, suggesting a close evolutionary relationship between them even though they perform very different functions.

### Characteristic structural features

Cryptochromes show an overall structural similarity to DNA photolyases (Figure 2a,b), despite the fact that cryptochromes possess no photolyase activity. Most cryptochromes, with the exception of CRY-DASH proteins, are composed of two domains, an amino-terminal photolyase-related (PHR) region and a carboxy-terminal domain of varying size [4] (Figure 2c). The PHR region of cryptochromes appears to bind two chromophores, cofactors that absorb light; one chromophore is flavin adenine dinucleotide (FAD) and the other 5,10-methenyltetrahydrofolate (pterin or MTHF) [9,12,13]. Photolyases also have FAD, and the second chromophore can be either pterin or deazaflavin [9,13]. The carboxy-terminal domain of cryptochromes is generally less conserved than the PHR region [2]; it is longer in most plant cryptochromes than animal cryptochromes, and CRY-DASH proteins lack this domain.

Crystal structures have been determined for three members of the photolyase/cryptochrome superfamily in various species: CPD photolyase (*Escherichia coli* (Figure 2b), *Synechococcus* sp. and *Thermus thermophilus*) [14], CRY-DASH (*Synechocystis* sp.; Figure 2d) [11], and a plant cryptochrome (the PHR region of *Arabidopsis* CRY1; Figure 2a) [15]. The PHR regions have remarkably similar three-dimensional structures: all three fold into two domains, an  $\alpha/\beta$  domain and a helical domain, which are connected by a variable loop that wraps around the  $\alpha/\beta$  domain (Figure 2c,d). The  $\alpha/\beta$  domain adopts a dinucleotide-binding fold with five-strand parallel  $\beta$  sheets flanked on either side by  $\alpha$  helices. Two lobes of the helical domain form a cavity called the FAD-access cavity in all three proteins [11,14,15]. FAD is buried deeply inside the FAD-access cavity, but it may be accessible to the solvent from the bottom of the cavity. FAD binds non-covalently to the protein in a U-shaped conformation, with its adenine and isoalloxazine rings positioned at the bottom of the cavity (Figure 2d).



**Figure 2**  
The structure of cryptochromes. A comparison of the structures of the photolyase-related (PHR) regions of (a) *Arabidopsis* CRY1 and (b) *E. coli* DNA photolyase. White lines indicate the boundaries of the FAD-access cavity; red and blue represent areas of negative and positive electrostatic potential, respectively. Reproduced with permission from [15]; copyright 2004 National Academy of Sciences USA. (c) A schematic representation of a typical photolyase/cryptochrome superfamily protein. The parts of the PHR region bound by pterin and FAD are indicated with brackets and the domains are shown below the protein. (d) The overall fold of a CRY-DASH protein (*Synechocystis* sp. PCC6803 cryptochrome).

Despite the overall structure similarity, the *Arabidopsis* CRY1 PHR region has several structural characteristics that differ from photolyase and CRY-DASH. First, photolyase

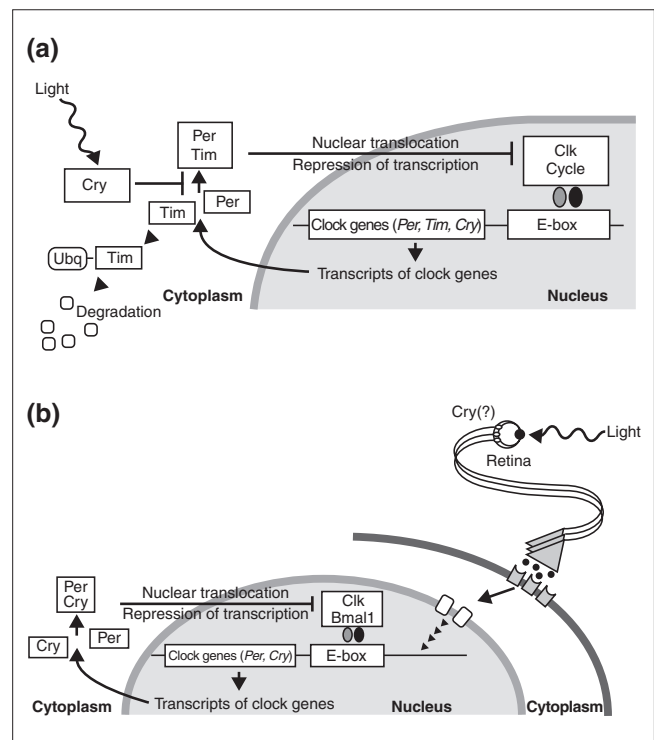
has a generally positively charged groove running through the FAD-access cavity (Figure 2b), which is where DNA interacts, and CRY-DASH has a similar positive electrostatic potential on the surface around the cavity, consistent with the observation that CRY-DASH binds DNA [11]. The CRY1 PHR region does not have this DNA-binding groove (Figure 2a), indicating that it may not bind DNA directly. Second, the surface of CRY1 PHR is predominantly negatively charged, except for a small concentration of positive charges near the FAD-access cavity [15]. Another distinction between the PHR regions of photolyase/CRY-DASH and CRY1 is that the FAD-access cavity of the latter is larger and deeper (see white line in Figure 2a). This difference is consistent with the fact that unlike photolyase, cryptochrome does not bind a pyrimidine dimer in the cavity. Finally, a single molecule of an ATP analog (AMP-PNP) can bind in the FAD-access cavity of the CRY1 PHR region [15], whereas neither photolyase nor CRY-DASH is reported to bind ATP.

### Localization and function

Circadian biological clocks are biochemical oscillators that cycle about every 24 hours and that can be reset (entrained) by exposure to light and other environmental signals. In animals there is a central oscillator in the brain that controls the circadian behavior of the whole organism as well as peripheral oscillators in some tissues. The oscillation arises from a transcriptional feedback loop involving a set of clock transcription factors, including timeless (Tim), period (Per), clock (Clk) and Bmal1 as well as cryptochromes. Cryptochromes are ubiquitously expressed in the organs and tissues of all organisms, and they are generally nuclear proteins that regulate gene expression. The best-studied animal cryptochromes are *Drosophila* cryptochrome Cry and mouse cryptochromes Cry1 and Cry2 [16-20], and the two *Arabidopsis* cryptochromes CRY1 and CRY2 have also been extensively studied.

#### *Drosophila* cryptochromes

*Drosophila* Cry is a predominantly nuclear protein that mediates regulation of the circadian clock by light [16,17], although it can also be found in the cytosol [21]. It regulates the circadian clock by interacting directly with the protein Tim to suppress the clock's negative feedback loop (Figure 3a). Light stimulates the Cry-Tim interaction, which promotes ubiquitination and proteasome-dependent degradation of Tim and represses formation of the Per-Tim heterodimer. Inhibition of a heterodimer of the Clock and Cycle proteins by the Per-Tim heterodimer is thus released and the phase of circadian oscillation is reset [21] (Figure 3a). Cryptochrome is apparently not the only photoreceptor that entrains the circadian clock in *Drosophila*, however. The behavioral rhythmicity of the *cry<sup>b</sup>* mutant fly, which lacks Cry function, can nevertheless be entrained in response to light unless the signal transduction by the visual



**Figure 3**

Regulation of the circadian clock by animal cryptochromes. **(a)** In *Drosophila*, Cry suppresses the negative feedback loop of the circadian clock by binding to Tim in a light-dependent manner; this results in the proteasome-dependent ubiquitin-mediated degradation of Tim (Ubq, ubiquitination) and thus inhibition of the action of the Per-Tim heterodimer. Without Cry, the Per-Tim heterodimer would enter the nucleus and inhibit the binding of clock-cycle proteins (Per, Clk and Bmal1) to the E-box in the promoters of clock genes, preventing their expression. **(b)** In mammals, cryptochromes are integral parts of the negative feedback loop. The Cry protein interacts with Per to repress the activity of the transcription factors Clk and Bmal1 and thus to repress transcription. Cryptochromes may also be involved in the photo-entrainment of the mammalian circadian clock; clock genes are known to be regulated in response to neural signals from the retina in response to light, but it is not yet clear whether this involves cryptochromes.

pigment is also eliminated [17]. In addition to its role as a photoreceptor for the entrainment of the central oscillator of *Drosophila*, Cry also has a light-independent role in the function of the peripheral circadian oscillator [22].

#### Mammalian cryptochromes

The two functions of *Drosophila* Cry - as a photoreceptor for the entrainment of the circadian clock together with visual pigments and as an integral component of the circadian oscillator protein complex - are also features of the mammalian cryptochromes. Mammalian cryptochromes are predominantly nuclear proteins [18,20], but they can also be found in the cytosol [23]. Like *Drosophila* Cry, mammalian cryptochromes perform both light-dependent and light-independent functions in the regulation of the circadian

clock. Several observations demonstrate the light-dependent role of mammalian Cry proteins. Knockout mice lacking one or both *Cry* genes have a reduced or abolished ability to induce expression of genes such as *per* and the protooncogene *c-fos* in response to light [18,24]. Moreover, the pupils of mutant mice lacking both *Cry1* and *Cry2* have reduced reflex responses to light [25].

On the other hand, the *cry1 cry2* double mutant mouse shows an apparently normal rhythmicity in light-dark cycling conditions, but it loses rhythmicity instantaneously and completely in free-running (always dark) conditions [19]. These observations indicate that the Cry proteins play an essential and light-independent function in the mammalian central circadian oscillator, and that cryptochromes are not the only photoreceptors mediating light control of the clock. The fact that cryptochromes are integral parts of the mouse central oscillator makes it almost impossible to test directly their role in the light entrainment of the clock. Nevertheless, it has been found that, somewhat analogous to the situation for *Drosophila*, the mouse *cry* mutant retains its ability to mediate light input unless the function of visual pigments is also disrupted at the same time. Triple-mutant mice carrying mutations of both cryptochromes together with a retinal-degenerative mutation are nearly arrhythmic under light-dark cycling conditions [24]. These results demonstrate that mammalian Cry proteins are indeed involved in regulation of the circadian clock by light, but their role in the light entrainment of the circadian clock is carried out redundantly by other photoreceptors. It seems clear now that the additional photoreceptors acting together with cryptochromes for the entrainment of the mammalian circadian oscillator are visual rod-cone opsins and the related protein melanopsin [26,27].

Like *Drosophila* Cry, mammalian cryptochromes interact physically with clock proteins, including the promoter-binding transcription regulators *Per*, *Clk* and *Bmal1* (Figure 3b). In contrast to *Drosophila* Cry, mammalian Cry proteins are components of the negative-feedback loop of the circadian clock (Figure 3b). The physical interaction of cryptochrome with other clock components affects their activity, interaction, degradation, or nuclear trafficking, and consequently alters the transcriptional regulation of the clock genes [28,29]. But the interaction between cryptochromes and other clock proteins such as *Per*, *Clk* and *Bmal1* appears not to be affected by light, suggesting that such interactions may not be the mechanism of photo-entrainment of the circadian clock, as they are in *Drosophila*. In addition to the direct regulation of transcription via physical interaction with promoter-binding transcription regulators, cryptochromes may also affect the circadian clock by participating in the regulation of histone modifications [30,31], but how this works remains to be elucidated.

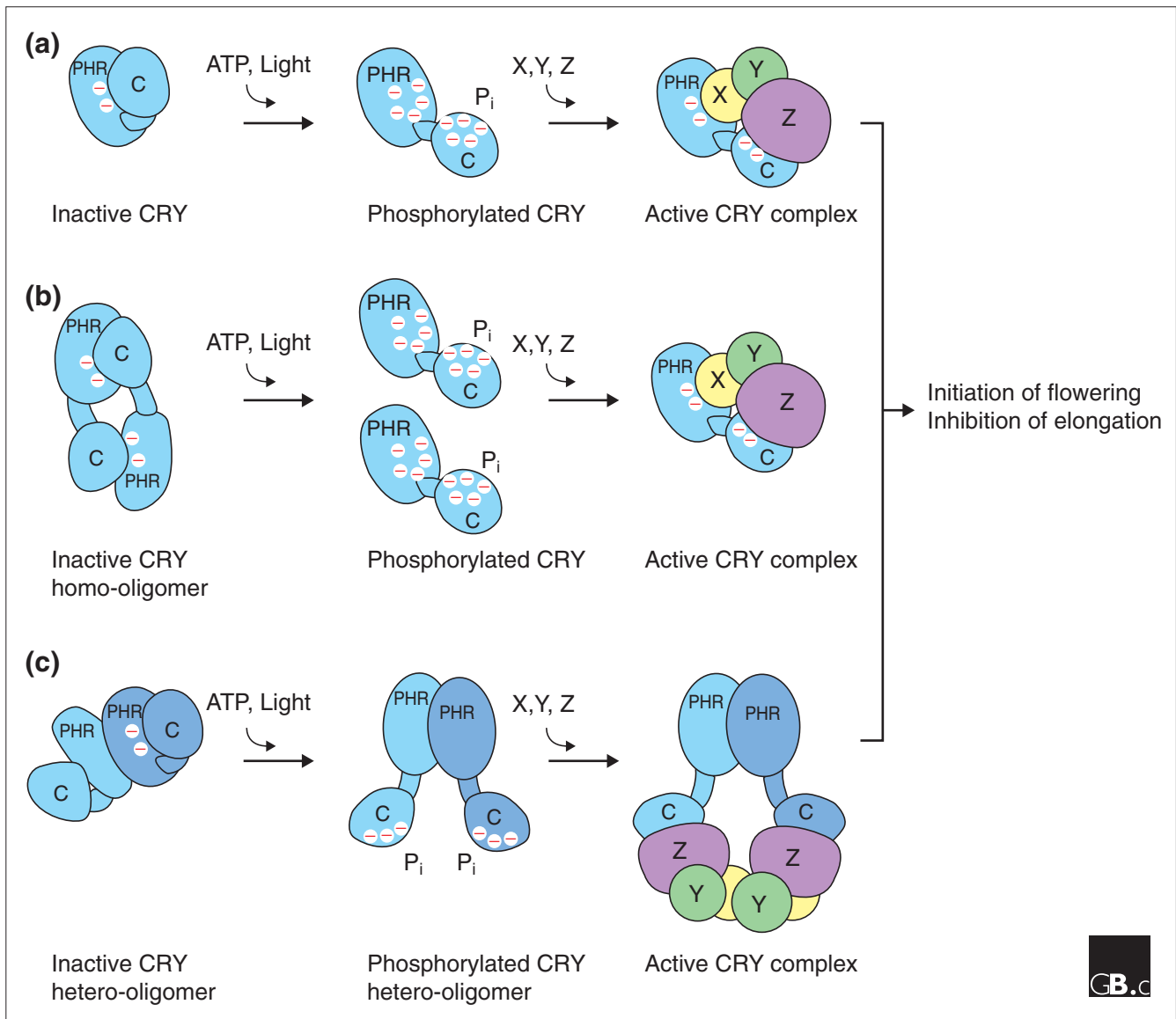
### **Arabidopsis cryptochromes**

*Arabidopsis* CRY1 and CRY2 are predominantly nuclear proteins that mediate regulation of gene expression and entrainment of the circadian clock in response to light [32-35]. CRY1 and CRY2 play major roles in plant photomorphogenesis, such as inhibition of stem elongation by blue light, stimulation of leaf expansion by blue light, and regulation of floral initiation by day length [6,36-38]. It appears that cryptochromes control developmental changes in plants via changes of gene expression in response to light. CRY1 and CRY2 together are responsible for blue-light-dependent changes in gene expression of up to 10-20% of the *Arabidopsis* genome [39].

There are at least two mechanisms by which cryptochromes may affect nuclear gene-expression changes in response to light. First, a cryptochrome molecule may interact with proteins associated with transcriptional machinery to affect transcription directly. *Arabidopsis* CRY2 binds to chromatin in a DNA sequence-independent manner ([40] and M. Maymon and C.L., unpublished observations), but it is unclear how a sequence-independent chromatin-interacting protein may regulate gene expression. Unlike the animal cryptochromes that have been shown to regulate transcription via physical interactions with promoter-binding transcription regulators, no such interaction has been reported for plant cryptochromes. An alternative model is that plant cryptochromes may interact with proteins exerting other cellular functions to regulate the stability, modification, cellular trafficking of the transcriptional regulators. For example, plant cryptochromes have been found to interact with an E3 ubiquitin ligase, COP1, suggesting that plant cryptochromes may act in the way not yet discovered for the animal cryptochromes [32,41,42]. Consistent with this view, it has also been found recently that *Arabidopsis* cryptochromes mediate suppression by blue light of the proteasome-dependent degradation of an important floral regulator, *CONSTANS* [43]. Exactly how cryptochromes do this needs to be investigated further.

### **Mechanism**

The catalytic mechanism of cryptochromes has not been fully elucidated, but some clues can be found in the mechanism of CPD photolyases, where FAD plays the main catalytic role [14]. In a DNA-repair reaction, CPD photolyase binds to the pyrimidine dimer of DNA and 'flips' it out from within the DNA duplex into the FAD-access cavity of the enzyme, to form a stable complex. The other chromophore (pterin or deazaflavin), which is also called the 'antenna' chromophore, absorbs photons of blue or UV-A light, and it transfers the excitation energy to the flavin of FAD. Flavin in the excited state donates an electron to the pyrimidine dimer to split the cyclobutane ring. The electron is transferred back to flavin in this process, resulting in regeneration of ground-state flavin. The repaired dinucleotide no longer fits into the FAD-access cavity, so it disassociates from the photolyase. The exact role of FAD and the FAD-access cavity in the

**Figure 4**

Possible models of the phosphorylation-dependent structural changes of plant cryptochromes in response to blue light. The PHR region is predominantly negatively charged (-), and the carboxy-terminal domain (C) can be made negatively charged by phosphorylation (which requires ATP and releases inorganic phosphate,  $P_i$ ). In all models, phosphorylation leads to binding of unknown signaling partners (X, Y, Z) and to regulation of plant development. **(a)** One model is that phosphorylation of the carboxy-terminal domain in response to light is performed by ATP bound to the PHR region; this leads to dissociation of the two domains. **(b)** A second possibility is that phosphotransfer in response to light involves the interaction of two cryptochromes encoded by the same gene. **(c)** Alternatively, intermolecular phosphotransfer could involve the interaction of different cryptochromes. All three scenarios may exist in plant cells, and the activity of a cryptochrome may be determined by the kinetics of the different reactions.

function of cryptochromes remains unclear, but it is conceivable that it may also be involved in electron-transfer reactions.

Although the PHR region that contains the chromophore(s) is the most conserved part of the proteins, the carboxy-terminal domain has been shown to have a role in the function or regulation of both animal and plant cryptochromes. Expression of the carboxy-terminal domains of *Arabidopsis* cryptochromes fused to the marker enzyme  $\beta$ -glucuronidase confers a

constitutive growth response to light even in darkness in the absence of the PHR region [32,41]. In contrast, the PHR regions of the *Drosophila* and *Xenopus* cryptochromes are physiologically active in the absence of the carboxy-terminal domain [44,45]. The carboxy-terminal domain of *Drosophila* Cry is important for protein stability, interaction with Tim, and sensitivity of the photoreceptor to circadian light signals [44], whereas the carboxy-terminal domain of *Xenopus* Cry is required for its nuclear localization [45].

Cryptochromes are regulated by phosphorylation. It has been shown that *Arabidopsis* cryptochromes are phosphorylated in response to blue light and that this is associated with the function and regulation of the photoreceptors [46,47]. Moreover, when *Arabidopsis* CRY1 was expressed in insect cells, it was found to undergo ATP-dependent and blue-light-dependent autophosphorylation [15,47,48]. It is not known whether animal cryptochromes also bind to ATP, although it has been shown that mouse cryptochromes are phosphorylated [49].

The interaction between the *Arabidopsis* CRY1 PHR region and ATP has a few interesting features reminiscent of the interaction between pyrimidine dimer and photolyase [14]: the phosphate groups of ATP are exposed to solvent; the adenine and ribose moieties are buried deep within the FAD-access cavity; and ATP can have a water-mediated contact with FAD [15]. The interaction of the *Arabidopsis* CRY1 PHR region with ATP also lacks several features commonly found in protein-ATP interactions, such as protein-to-phosphate interaction, protein-to-Mg<sup>2+</sup> contact, and a nearby serine residue for phosphotransfer [15]. An examination of the topology of the CRY1 PHR region structure shows, however, that all these features could potentially be provided by the carboxy-terminal domain of the cryptochrome (Figure 4). The observation that the serine-rich carboxy-terminal domains of *Arabidopsis* cryptochromes fused to  $\beta$ -glucuronidase are constitutively phosphorylated *in vivo* [32,46] suggests that a phosphotransfer may occur from ATP bound to the FAD-access cavity to the nearby carboxy-terminal domain (Figure 4a). It is also conceivable that photon-excited FAD may trigger electron transfer to the nucleotide and phosphotransfer from ATP to serine residues on the carboxy-terminal domain. Because the surface of the PHR region is predominantly negatively charged, especially in the place where the carboxy-terminal domain is likely to interact with it, the phosphorylated carboxy-terminal domain would then be repelled from the PHR region surface, resulting in a change of cryptochrome conformation. This conformational change would allow it to interact with other signaling proteins and to propagate the light signal (Figure 4a). Alternatively, another molecule of cryptochrome binding to the FAD-access cavity may also provide the missing features needed for a productive ATP-cryptochrome interaction. Indeed, both CRY2-CRY2 interaction and CRY1-CRY2 interactions can be detected in *Arabidopsis* (D. Shalitin, X. Yu, and C.L., unpublished observations). Formation of either a homo-oligomer or a hetero-oligomer of cryptochromes would provide a mechanism for intermolecular phosphotransfer, which may change the structure of the cryptochromes (Figure 4b,c).

## Frontiers

Despite recent progress in the study of cryptochromes, there are still many intriguing questions about their structure and mechanism. For example, what is the role of the FAD-access

cavity in cryptochrome? Can a photon-excited FAD trigger a phosphotransfer from ATP to the carboxy-terminal domain? Elucidation of the structure of holocryptochromes including the carboxy-terminal domain may help us to answer these questions. For mammalian cryptochromes, it is unclear whether the interaction of Cry with other clock proteins is affected by light *in vivo*, and also how may cryptochromes mediate regulation of the circadian clock by light. The physiological function of CRY-DASH proteins is also not clear, although they may regulate gene expression directly. Elucidation of the function and mechanism of CRY-DASH would provide additional clues about the evolutionary history of the cryptochromes.

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