

## RESEARCH ARTICLE

# Blue-light-receptive cryptochrome is expressed in a sponge eye lacking neurons and opsin

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### SUMMARY

Many larval sponges possess pigment ring eyes that apparently mediate phototactic swimming. Yet sponges are not known to possess nervous systems or opsin genes, so the unknown molecular components of sponge phototaxis must differ fundamentally from those in other animals, inspiring questions about how this sensory system functions. Here we present molecular and biochemical data on cryptochrome, a candidate gene for functional involvement in sponge pigment ring eyes. We report that *Amphimedon queenslandica*, a demosponge, possesses two cryptochrome/photolyase genes, *Aq-Cry1* and *Aq-Cry2*. The mRNA of one gene (*Aq-Cry2*) is expressed *in situ* at the pigment ring eye. Additionally, we report that *Aq-Cry2* lacks photolyase activity and contains a flavin-based co-factor that is responsive to wavelengths of light that also mediate larval photic behavior. These results suggest that *Aq-Cry2* may act in the aneural, opsin-less phototactic behavior of a sponge.

Key words: Porifera, cryptochrome, evolution, eye, vision.

### INTRODUCTION

Like many sponge larvae, *Amphimedon queenslandica* parenchymellae (flagellated free-living larvae) are capable of detecting differing light intensities for use as settlement cues (Leys et al., 2002). This phototactic ability is conferred by posterior, concentric rings of cells that are pigmented and/or possess long cilia that act as light-responsive ‘rudders’ to steer the animal, with maximal sensitivity near 440 nm (blue) (Leys et al., 2002). With pigment adjacent to photoreceptors, the sponge ring structure meets a minimal definition of an eye (Arendt, 2001). In all non-poriferan animals examined to date, including cnidarians (Koyanagi et al., 2008; Kozmik et al., 2008), eyes utilize photo-sensitive opsin proteins expressed in neurons that communicate information about the light environment to the nervous system. However, despite possessing many other G-protein coupled receptors (GPCRs), the fully sequenced genome of *A. queenslandica* lacks opsin, defined as opsin-clade GPCRs that possess a conserved Schiff base lysine that is central to opsin function (Plachetzki et al., 2007; Srivastava et al., 2010). Like other sponges, *A. queenslandica* lacks a nervous system. Therefore, *A. queenslandica* pigment ring eyes likely evolved convergently in the absence of opsins and nervous systems, and probably use as-yet-unknown molecular mechanisms that are fundamentally different from those employed by other animal eyes. One step in understanding the function of these convergently evolved sponge eyes is to identify molecular components and their functions.

The behavioral action spectrum of sponge light sensitivity led previous authors (Leys et al., 2002) to suggest that a flavin-based

molecule such as a cryptochrome, acts as the photopigment. Cryptochromes maximally absorb blue light and have light-mediated behavioral roles in multiple animal lineages. For example, *Drosophila* and mouse cryptochromes are involved in circadian rhythm entrainment (reviewed in Sancar, 2004), coral cryptochrome mRNA is upregulated during spawning, which occurs at the full moon (but see Hoadley et al., 2011; Levy et al., 2007), and several animal cryptochromes have been implicated in magnetoreception (Foley et al., 2011; Gegeer et al., 2008; Gegeer et al., 2010; Mouritsen et al., 2004). This previous research led us to examine cryptochrome as a candidate gene for use in the aneural eyes of *A. queenslandica*.

Here we determine gene expression patterns and the *in vitro* absorption spectra of sponge cryptochrome proteins for comparison with previously published behavioral action spectra. We report that *A. queenslandica* possesses two cryptochrome/photolyase genes with strikingly complementary expression. The mRNA of one gene (*Aq-Cry2*) is expressed in cells of the pigment ring. We show that the encoded protein lacks DNA photolyase activity *in vitro* and contains a flavin-based co-factor, and that the absorbance peak of the molecule matches the peak activity of light-mediated larval behaviors. These results suggest that *Aq-Cry2* functions in an evolutionarily convergent, neuron-less, opsin-less eye.

### MATERIALS AND METHODS

#### Bioinformatics

We first performed BLASTP searches of *Amphimedon queenslandica* Hooper and Van Soest 2006 gene models using

known cryptochromes and, based on overall similarity, we found two putative photolyase/cryptochrome genes in the sponge. We compared the phylogenetic relationships of the two newly determined sponge genes (*AqCry1* and *AqCry2*) to previously characterized photolyase/cryptochrome genes. First, we included in the phylogeny all previously known poriferan genes, including *Aphrocallistes vastus* photolyase related protein (CAD24679.1), *Crateromorpha meyeri* cryptochrome (CAZ66368.1) and *Suberites domuncula* cryptochrome (CAZ66367.1). Next, we included the cnidarian cryptochrome genes previously investigated and analyzed by Reitzel et al. (Reitzel et al., 2010), including four *Nematostella vectensis* genes (XP\_001623146.1, XP\_001631029.1, XP\_001632849.1 and XP\_001636303.1) and three *Acropora millepora* genes (ABP97099.1, ABP97098.1 and the translated EST sequence DY585180). To these, we added genes that represent the major clades of the photolyase/cryptochrome family, as defined by Brudler et al. (Brudler et al. 2003): plant cryptochromes (*Arabidopsis thaliana* CRY2, Q96524.2; *Arabidopsis thaliana* CRY1, Q43125.2); class 2 photolyases (*Arabidopsis thaliana* AF053365.1); DASH cryptochromes (*Arabidopsis thaliana* AB062926.1); class 1 photolyases (*Escherichia coli* P00914.1); and representatives of the major clades of the animal cryptochrome/6-4 photolyase family, including insect type-2 cryptochromes (*Apis mellifera* NP\_001077099.1), vertebrate cryptochromes (*Mus musculus* Cry2 CAM15994.1), 6-4 photolyases (*Drosophila melanogaster* BAA12067.1) and insect type-1 cryptochromes (*D. melanogaster* CRY AAF55649.1).

Using each of the proteins listed above (excluding poriferan and cnidarian sequences) as 'bait' for database searches, we expanded the representation of genes in the phylogenetic analysis employing a pipeline of shell and perl scripts (Rivera et al., 2010; Tong et al., 2009). Each bait sequence from a major photolyase/cryptochrome clade was used to perform similarity searches using BLASTP (Altschul et al., 1997) of two non-redundant protein databases, UniRef50 and UniRef90, curated by UniProt (<http://www.uniprot.org/>). For each bait protein, the two most similar genes in the UniRef50 and the 15 most similar genes in the UniRef90 database were retained for phylogenetic analyses. Identical sequences, such as those obtained from both UniRef90 and UniRef50 databases, were removed from further analysis.

We next aligned together all poriferan, cnidarian, database-retained and bait sequences using MUSCLE (Edgar, 2004). Then we estimated maximum likelihood (ML) phylogenetic trees using 10 Slow ML searches in RAXML, assuming a WAG (Stamatakis, 2006) model of protein evolution. Nine of 10 searches yielded the same likelihood value, suggesting that the algorithm located the global maximum. We gauged consistency of the phylogeny using 100 fast bootstrap pseudoreplicates, also implemented in RAXML (Stamatakis et al., 2008). We visualized the resulting phylogenetic trees with TreeView (Krauer et al., 1996) or FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

#### Construct preparation

After finding two candidate *A. queenslandica* cryptochrome genes by bioinformatic analyses, we cloned both genes for subsequent *in vitro* expression, purification and biochemical analyses. First, we cloned *Aq-Cry 1* into pCR vector (Invitrogen, Carlsbad, CA, USA) using the primers 5'-ATGGAGACTCTACAAGGCTCCTACG-3' (forward) and 5'-CTATGGATTATCACCATATAGTCCCT-3' (reverse) on a cDNA library. For subcloning into an expression vector (pMALc2; New England Biolabs, Ipswich, MA, USA), we added *Hind*III sites to the primers and the resultant fragment was

cut and cloned into the *Hind*III site of pMALc2 (pMALc2-CRY1). In addition, we synthesized *Aq-Cry2* in pMALc4X (pMALc4X-CRY2; New England Biolabs) with GeneOnDemand (GenScript, Piscataway, NJ, USA). Both pMALc2-CRY1 and pMALc4X-CRY2 are maltose binding protein (MBP) fusions, making MBP-AqCry1 and MBP-AqCry2 proteins, respectively. MBP-AqCry1 and MBP-AqCry2 were purified from the photolyase-deficient *Escherichia coli* strain UNC523 and were used for *in vitro* photolyase activity assays. We also cloned the genes into expression plasmids constructed with His tags for Sf21 insect cell-based protein purification, which yield cleaner protein samples for spectroscopic studies. We amplified *Aq-Cry1* cDNA from pMALc2-CRY1 using the oligos 5'-ACAGAATTCGCCACCATGGAGACTCTACAAGGCTCCTAC-3' (forward) and 5'-AGTCTAG-AATAAAGTCTACAAAAAATCCGGAA-3' (reverse). We amplified *Aq-Cry2* cDNA from pMALc4X-CRY2 using the oligos 5'-GCGAATTCGCCACCATGGACAGCTATAGCAATGGTTTC-3' (forward) and 5'-GAATCTAGAGGGGTTTGTGGATG-CGAC-3' (reverse), and inserted the resulting PCR products into the *Eco*RI and *Xba*I sites of pAc5.1V5/HisA (Invitrogen) to make pAc5.1AqCRY1-V5/HisA and pAc5.1AqCRY2-V5/HisA, respectively. The coding sequence from this was cut using *Eco*RI and *Pme*I and inserted into the *Eco*RI and *Stu*I sites of pFast-Bac1 (Invitrogen) to make pFast-Bac1-AqCRY1-V5/HisA and pFast-Bac1-AqCRY2-V5/HisA.

#### Protein purification

We prepared baculovirus using the Gibco BRL Bac-to-Bac baculovirus expression system (Invitrogen). Briefly, pFast-Bac1-AqCRY2-V5/HisA was transformed into the *E. coli* DH10Bac, and recombinant bacmids were isolated from 1 ml of bacterial cultures grown from colonies of transformants. We transfected Sf21 cells in six-well plates with 1 µg of the bacmid using 6 µl of Cellfectin reagent (Invitrogen), and parental virus was collected after 72 h. After two more 72 h amplification steps, we obtained the third passage (P3), high titer stock, and used it to infect Sf21 cells at a large scale. We infected 1 liter of Sf21 cells ( $10^6$  ml<sup>-1</sup>) growing at 27°C in spinner flasks with the P3 high titer virus at a ratio of 1:100 (v/v); the cells were harvested 2 days later. Cells were resuspended in 5 ml of 1× Tris-buffered saline (TBS; pH 7.6) containing 20 mmol l<sup>-1</sup> imidazole, and sonicated 10×10 s with a Branson sonicator (Branson Ultrasonics, Danbury, CT, USA). The cell debris was removed by centrifugation at 4°C for 90 min in a Ti 45 rotor at 137,000 g in a Beckman L-80 centrifuge (Palo Alto, CA, USA). Cell free extract (30 ml) was incubated with 0.5 ml of Ni-NTA agarose beads (Qiagen, Valencia, CA, USA) for 3 h. Then we washed the beads four times with 15 ml of TBS containing 20 mmol l<sup>-1</sup> imidazole to remove unbound proteins. AqCRY2V-5/HisA protein was eluted from beads by increasing the imidazole to 200 mmol l<sup>-1</sup>. We dialyzed the eluted protein against the storage buffer containing 50 mmol l<sup>-1</sup> Tris-HCl, pH 7.5, 100 mmol l<sup>-1</sup> NaCl, 5 mmol l<sup>-1</sup> dithiothreitol, and 50% (v/v) glycerol and kept the eluted protein at -20°C. We analyzed the purity of recombinant protein by performing electrophoresis on a NuPAGE 4–12% Bis-Tris Gel followed by Coomassie Blue staining. The yield was 3 mg of recombinant protein from 1 liter of Sf21 culture. AqCRY1V-5/HisA protein was made in the same way.

Preparation of three photolyase controls, each with MBP fused at the N terminus, has been described previously: *Arabidopsis* Cry (Selby and Sancar, 2006), *Xenopus laevis* 6-4 photolyase (Kim et al., 1996; Zhao et al., 1997) and *Caulobacter crescentus* photolyases (Ozturk et al., 2008). We purified MBP fusion proteins of *Aq-Cry1*

and *Aq-Cry2* according to the manufacturer's recommendations (New England Biolabs). Briefly, transformed UNC523 (photolyase deficient) bacterial cells were grown in 1 liter of Luria-Bertani (LB) medium at room temperature. At an OD<sub>600</sub> (the optical density measured at a wavelength of 600 nm) of 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mmol l<sup>-1</sup>, and growth continued for 5 h. We washed the cell pellets and resuspended them in 30 ml of column buffer (20 mmol l<sup>-1</sup> Tris, pH 7.4, 200 mmol l<sup>-1</sup> NaCl, 1 mmol l<sup>-1</sup> EDTA, 10 mmol l<sup>-1</sup> 2-mercaptoethanol) before sonicating 10 times for 10 s each and pelleting. Supernatants were applied to columns containing 20 ml of amylose resin (New England Biolabs). We washed the columns with five volumes of column buffer and then eluted with 20 mmol l<sup>-1</sup> maltose in column buffer. Fractions were analyzed on polyacrylamide gels, pooled and dialyzed against 50 mmol l<sup>-1</sup> Tris, pH 7.5, 100 mmol l<sup>-1</sup> NaCl, 1 mmol l<sup>-1</sup> EDTA, 5 mmol l<sup>-1</sup> dithiothreitol (DTT), 50% glycerol.

#### ***In vivo* photolyase activity assay**

We expressed *Aq-Cry1* or *Aq-Cry2* in bacteria that are not competent to repair UV-induced lesions in their DNA, to test whether heterologously expressed proteins rescue repair activity. We transformed competent SY2 cells [UV-sensitive strains *phr*-, *recA*- and *uvrA*- (Sancar et al., 1984)] with pMALc4X-CRY2, pMALc2-CRY1, pMALc4X (empty expression vector) or positive control pMS969, which expresses *E. coli* photolyase (Sancar et al., 1983). We grew these four SY2 strains to OD<sub>600</sub>=1.0 in Luria-Bertani ampicillin (LB-Amp) medium and 1 mmol l<sup>-1</sup> IPTG was added for 4 h to induce expression. We spun down 1.5 ml of culture and resuspended in 1 ml of 1% NaCl (saline). This was added to an additional 9 ml of saline on a Pyrex® Petri dish with the cover underneath to add an additional layer of UVC protection (Lepre et al., 1998). We then placed these on an FB-TIV 816 UV Transilluminator (Fisher Scientific, Pittsburg, PA, USA) at 20% intensity with cellulose acetate as an additional UVC filter for 1 h. Forty-microliter aliquots of each saline suspension were taken and added to 100 μl of LB-Amp + 1 mmol l<sup>-1</sup> IPTG and plated on LB-Amp plates. We counted colonies by hand after growing overnight in ambient light. We found that positive control cells expressing *E. coli* Phr (a photolyase) grew to a high density, with longer UV exposures resulting in lower density of growth. Results with cells heterologously expressing each *Aq-Cry* varied, with each protein leading to cell growth in at least one trial (data not shown). This may be explained by the known prevalence of false positives using this method (Malhotra et al., 1995).

#### ***In vitro* photolyase activity assay and photoreduction**

We generated radioactive oligos with a cyclobutane thymine dimer, T<math>\rightleftharpoons</math>T or (6-4) photoproduct, T[6-4]T, within an *MseI* cut site (TTAA). The photoproducts block digestion by *MseI*; photo-repair regenerates the restriction site. Single-stranded substrates were prepared as described previously (Selby and Sancar, 2006). To make double-stranded oligos, <sup>32</sup>P-radiolabeled oligonucleotide with photoproduct and complementary oligonucleotide at 1:20 ratio were heated to 85°C for 5 min in an annealing buffer containing 50 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> Tris-HCl pH 7.5 and 10 mmol l<sup>-1</sup> MgCl<sub>2</sub>, and then cooled slowly to 25°C. Repair reactions (20 μl) contained 50 nmol l<sup>-1</sup> Tris-HCl, pH 7.5, 100 mmol l<sup>-1</sup> NaCl, 1 mmol l<sup>-1</sup> EDTA, 10 mmol l<sup>-1</sup> DTT, 0.2 nmol l<sup>-1</sup> substrate, and the indicated concentrations of enzyme purified from UNC523 cells. Reactions were incubated in the dark at 25°C for 15 min and then exposed to 366 nm light at a fluence rate of 2 mW cm<sup>-2</sup> for 60 min

from two black light lamps (F15J8-BLB; General Electric, Fairfield, CT, USA), filtered through a glass plate to cut out short wavelength light under 300 nm, at 4°C (dark samples were covered with aluminum foil). Following photoreactivation, the DNA was extracted with phenol, precipitated with ethanol, resuspended in 40 μl of buffer containing 10 mmol l<sup>-1</sup> Tris-HCl, pH 7.9, 50 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> MgCl<sub>2</sub>, and 1 mmol l<sup>-1</sup> DTT, heated to 85°C for 5 min and then cooled slowly to 25°C. To this, 40 units of *MseI* were added and digestion was carried out at 37°C overnight. Products were analyzed on 8% polyacrylamide sequencing gels followed by autoradiography. We quantified data from degradation and *MseI* digestion (repair) assays by densitometry using ImageQuant 5.0 software (Molecular Dynamics, Waukesha, WI, USA).

To quantify the absorption spectrum of *Aq-Cry1* and *Aq-Cry2*, we grew His-tagged *Aq-Cry1* and *Aq-Cry2* in insect Sf21 cells and purified them using nickel agarose beads, as described above, under yellow light to prevent photochemical modification (Song et al., 2007). We used insect cells rather than bacterial cells as they allow for purification of enzymes with their flavin adenine dinucleotide co-factor. Purified protein in storage buffer was irradiated with 366 nm light at a fluence rate of 2 mW cm<sup>-2</sup> for 15 min using an F15T8-BLB black light (General Electric) lamp as a light source. We performed the same procedure on *Aq-CRY1*-His purified from Sf21 cells and purified MBP-*AqCry1*, which showed the same absorption spectrum. We recorded absorption spectra using a Shimadzu UV-1601 spectrophotometer (Columbia, MD, USA).

#### ***In situ* hybridization**

We made templates for *in situ* probes by designing primers against predicted coding sequences and EST sequences (Srivastava et al., 2010). We made an *in situ* probe for *Aq-Cry1* by amplifying a 1166 bp fragment from cDNA using the primers R1261 (5'-GCAACCCACCGTAGTCTTGT-3') and L108 (5'-CCTTCCCAATGATGCAGTTT-3'). This was cloned into TOPO-pCR II (Invitrogen) using the manufacturer's protocol. We cloned a 1232 bp fragment of *Aq-cry2* in the same way using the primers R1487 (5'-GAGATTGCCTGTCAGCCTC-3') and L215 (5'-TAGTCTCCGTTTGGGTCCAG-3'). All clones were sequenced for verification using M13F and M13R primers. Digoxigenin (DIG)-labeled antisense RNA probes were transcribed from polymerase chain reaction (PCR) products using DIG RNA Labeling Mix (Roche Applied Sciences, Indianapolis, IN, USA) and T7 or SP6 Polymerase (Promega, Madison, WI, USA) following the manufacturer's instructions. We obtained embryonic and larval material from Heron Island Reef according to the procedures described in Leys et al. (Leys et al., 2008). We performed *in situ* hybridization and subsequent processing following previously published protocols (Larroux et al., 2008). We observed whole-mount and sectioned samples under an Olympus SZH or BX60 microscope with Nomarski optics (Olympus Australia Pty Ltd, Mt Waverly, VIC, Australia) and photographed samples with a Nikon Sight DS-U1 camera (Nikon Australia Pty Ltd, Lidcombe, Australia).

## **RESULTS**

### **Identification of two cryptochromes from *A. queenslandica***

Because *A. queenslandica* larvae are responsive to wavelengths of light overlapping the flavin response spectrum (Leys et al., 2002), we identified poriferan photolyase/cryptochrome orthologs as candidate genes mediating larval phototactic behaviors. Photolyases are ancient enzymes present in both prokaryotes and eukaryotes that function in blue-light-driven repair of UV-induced DNA lesions. Cryptochromes are members of this family, but lack DNA repair

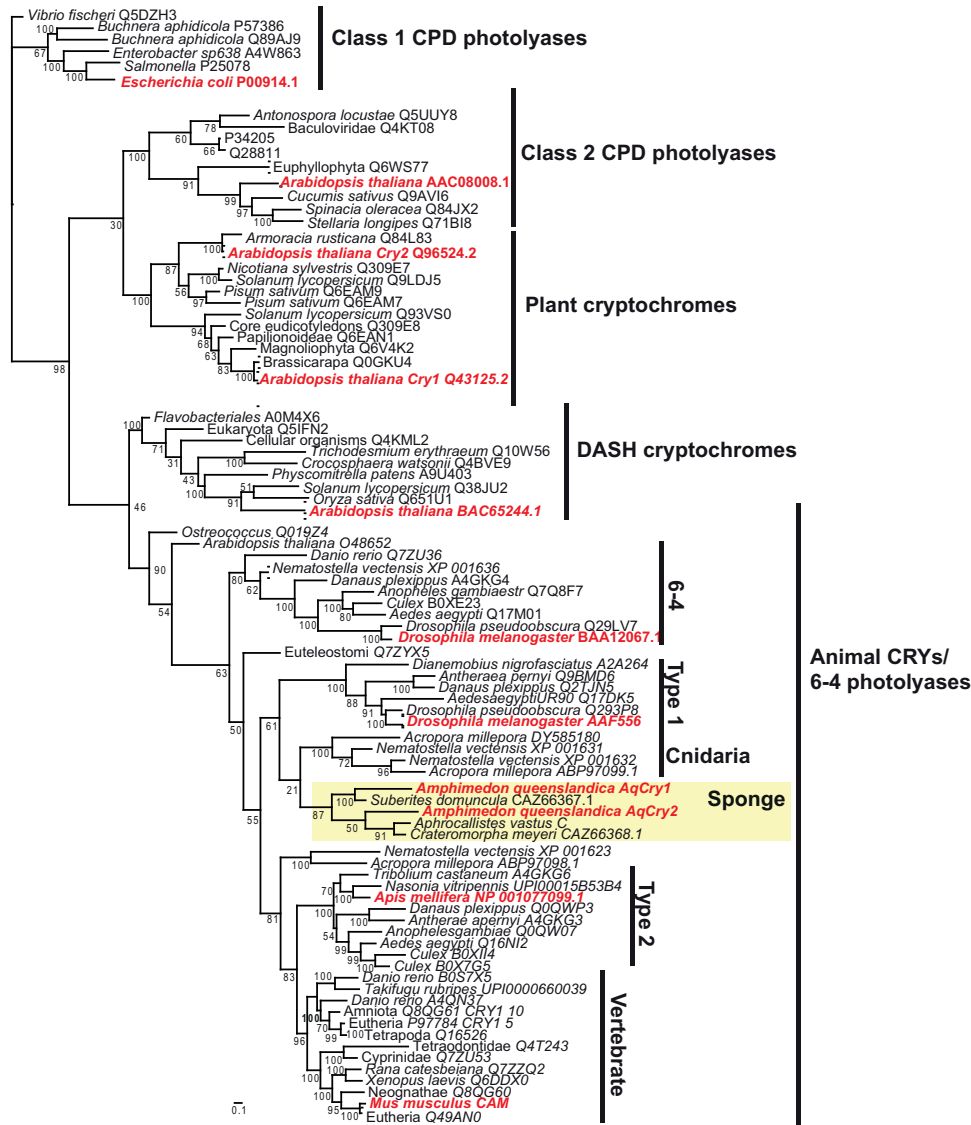


Fig. 1. Phylogenetic analysis of sponge cryptochromes (*Amphimedon queenslandica* Aq-Cry1 and Aq-Cry2), cnidarian cryptochromes/photolyases and previously defined major clades of the cryptochrome/photolyase family. We selected representatives of major clades (in red) and used them as 'bait' to find similar sequences in UniRef databases. We next aligned together all poriferan, cnidarian, database-retained and bait sequences using MUSCLE. Then we estimated maximum likelihood phylogenetic trees using 10 Slow ML searches in RAxML, assuming a WAG model of protein evolution. Numbers at nodes represent percentages of 100 fast-bootstrap pseudoreplicates containing that node. CPD, cyclobutane pyrimidine dimer.

functions, instead acting as blue-light receptors utilizing two chromophores: flavin and pterin (Sancar, 2000; Selby and Sancar, 2006). In animals, cryptochromes are involved in light-mediated behaviors, including the entrainment of circadian rhythms.

Using similarity and phylogenetic analyses, we identified and cloned two photolyase/cryptochrome genes using the fully sequenced genome of *A. queenslandica* (Srivastava et al., 2010) and designated them *Aq-Cry1* and *Aq-Cry2*. These genes, *Aq-Cry1* and *Aq-Cry2*, together with previously characterized genes from two hexactinellid sponges and another demersponge, form a clade of poriferan cryptochromes (Fig. 1). *Aq-Cry1* is most closely related to another demersponge cryptochrome, suggesting that the *Aq-Cry1* and *Aq-Cry2* originated by duplication some 650–800 million years ago (Sperling et al., 2010), prior to the origin of demersponges, but after the divergence of hexactinellids and demersponges. The poriferan cryptochrome genes are part of a large clade of mostly animal photolyase/cryptochromes that includes the animal cryptochromes (Abushik et al., 1985) and 6-4 photolyases. Activity in response to light varies across this group of proteins with respect to proteolytic cleavage, flavin radical formation and downstream targets (Ozturk et al., 2009; Sancar, 2004; Yuan et al., 2007; Zhu et al., 2005). Although sampling is incomplete, many members,

namely the 6-4 photolyases, exhibit light-mediated DNA repair activity.

To begin to elucidate the molecular function of sponge cryptochromes, we tested their ability to repair UV-damaged DNA, the defining feature of photolyases. Although all bona fide cryptochromes examined lack photolyase activity (Selby and Sancar, 2006), the sponge cryptochromes form a sister group to a clade of genes containing both cryptochromes and photolyases and thus may be able to act as photolyases. Furthermore, Schröder et al. (Schröder et al., 2003) concluded that the photolyase/cryptochrome protein of the hexactinellid sponge *Aphrocallistes vastus* possesses repair activity. We first tested for repair activity using the same assay as in Schröder et al. (Schröder et al., 2003). This assay was inconclusive, giving mixed results for both genes (data not shown; see Materials and methods for details), possibly because of the high false-positive rate of this assay (Malhotra et al., 1995).

To more rigorously test for photolyase activity in the *A. queenslandica* cryptochromes, we performed *in vitro* assays testing for the repair of a 6-4 photoproduct thymine dimer and a cyclobutane pyrimidine dimer (CPD). We found that neither sponge cryptochrome was able to repair (6-4) photoproducts,

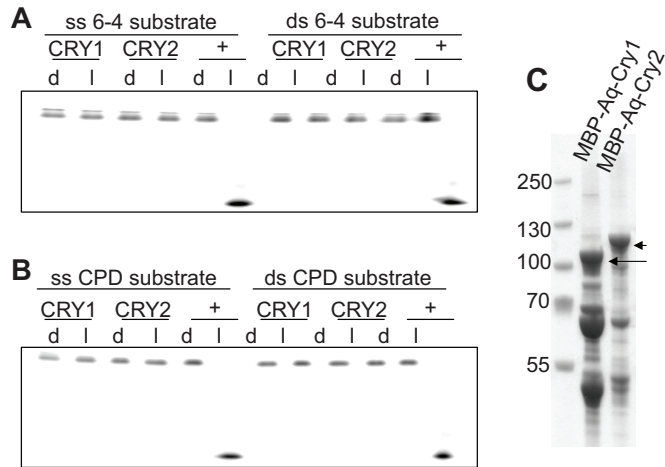


Fig. 2. DNA repair ability of *A. queenslandica* cryptochromes. A 48-nt-long radiolabeled oligomer with photoproducts in the TTAA sequence (*MseI* recognition site) in the center, in single-stranded (ss) or double-stranded (ds) form, was mixed with the appropriate enzyme and exposed to photoreactivating light (l) or kept in the dark (d). The level of repair was determined by the susceptibility of the DNA to cleavage by *MseI* endonuclease. Repaired products were annealed with the complementary strand (1:100 fold) before digestion with *MseI*. The reaction products were separated on 8% polyacrylamide gels. (A) Single- and double-stranded oligos containing CPD dimers were incubated in  $100 \text{ nmol l}^{-1}$  of *Aq-Cry1*, *Aq-Cry2*, and positive controls *Arabidopsis* DASH photolyase (ss substrate) and *Caulobacter crescentus* photolyase (ds substrate) for 90 min in the dark or  $2 \text{ mW cm}^{-2}$  black light. Oligos with repaired photoproducts became sensitive to *MseI* digestion, resulting in a shorter oligo. In these conditions, *Aq-Cry1* and *Aq-Cry2* did not repair photoproducts, indicating that they do not have any photolyase activity *in vitro*. (B) Single- and double-stranded oligos containing [6-4] photoproducts were incubated in  $100 \text{ nmol l}^{-1}$  of *Aq-Cry1*, *Aq-Cry2*, or positive control *Xenopus* 6-4 photolyase (ss and ds substrate) for 90 min in the dark or  $2 \text{ mW cm}^{-2}$  black light. Oligos with repaired photoproducts became sensitive to *MseI* digestion, resulting in a shorter oligonucleotide. In these conditions, *Aq-Cry1* and *Aq-Cry2* did not repair photoproducts, indicating that they do not have any photolyase activity *in vitro*. (C) Analysis of purified protein by 4–12% gradient SDS-PAGE. The numbers on the left indicate size markers. Lane 1, purified MBP-AqCRY1; lane 2, purified MBP-AqCRY2 through amylose resin. The positions of full-length CRYs are indicated by arrows. Extensive washing of the amylose resin by high-salt buffer causes considerable loss of the flavin co-factor. Therefore, the proteins bound to amylose resin were washed with two column volumes of buffer with moderate ionic strength before elution with maltose, and the preparation contains contaminating proteins at a considerable level. However, because the recombinant proteins were expressed in the photolyase-deficient *E. coli* strain UNC523 (phr::kan uvrA::Tn10), the contaminating proteins do not interfere with subsequent *in vitro* photoreactivation assays.

indicating that neither has (6-4) photolyase activity (Fig. 2A). Similarly, neither CRY exhibited CPD photolyase activity (Fig. 2B), demonstrating that they are cryptochromes rather than photolyases.

#### Expression of *Aq-Cry1* and *Aq-Cry2* show complementary patterns

Having established that the *Aq-Cry1* and *Aq-Cry2* are indeed cryptochromes, by phylogenetic position and lack of photolyase activity, we next examined whether either gene was expressed in a pattern suggesting involvement with phototactic behaviors. To this end, we performed *in situ* hybridizations across developmental stages to reveal patterns of gene expression.

The two *A. queenslandica* cryptochromes show strikingly complementary expression patterns in *in situ* hybridization experiments with *Aq-Cry2*, but not *Aq-Cry1*, expressed around the pigment ring (Figs 3, 4). Early expression of both genes is similar and is seen in the medium-sized cells scattered throughout the embryo, and *Aq-Cry1* transcripts are found in maternal follicle cells around the developing embryo (Fig. 3A,B, Fig. 4A,B). For descriptions of *A. queenslandica* development, see Adamska et al., Leys et al., and Leys and Degnan (Adamska et al., 2007; Leys et al., 2002; Leys and Degnan, 2001). During the ‘spot stage’ of embryonic development, pigment cells coalesce to form a pigmented spot at the posterior pole of the embryo. At this stage, *Aq-Cry2* transcripts are strongly enriched directly adjacent to and around the pigmented cells (Fig. 4C–E). In contrast, during this spot stage, we observed *Aq-Cry1* expression in cells restricted to the outer layer of the embryo, with no expression detected in the vicinity of the pigment ring (Fig. 3C,D). Later during development, as the pigment cells form the ring-eye, *Aq-Cry2* expression remains largely restricted to cells adjacent to, underneath and inside the ring, although transcripts are also detected in cells comprising an epithelial-like layer (Fig. 4F,G). In contrast, during ring formation, *Aq-Cry1* expression is weaker in the future photosensitive cells around the pigment ring (Fig. 3E–G). These expression patterns are maintained until larval hatching (Fig. 3H,I, Fig. 4H–J). After hatching, the expression of both cryptochrome genes becomes more diffuse and *Aq-Cry2* is no longer enriched around the pigment ring, though it still shows expression in pigment cells (Fig. 3J–M, Fig. 4K,L).

#### *Aq-Cry2* is responsive to blue light but does not undergo proteolysis

We next examined the *in vitro* absorption spectrum of *Aq-Cry2* and found similarities to the action spectrum of *A. queenslandica* larval photo-behaviors. The absorption spectrum of fully oxidized, purified *Aq-Cry2* ranges between 366 and 450 nm and is typical of enzyme-bound oxidized flavin adenine dinucleotide (FAD) (Fig. 5A,C), and the ratio of co-factor to apoprotein shows that recombinant *Aq-Cry2* contained stoichiometric flavin (Fig. 5A) (Worthington et al., 2003). Upon exposure to UV light, *Aq-Cry2* showed a typical type-1 cryptochrome reduction pattern (Fig. 5C), being converted to FAD ionic radical (Ozturk et al., 2009). The absorption peak of *Aq-Cry2* near 450 nm corresponds well with the primary, dominant peak of the *A. queenslandica* behavioral action spectrum (Leys et al., 2002). The lack of activity at 400 nm in the behavioral action spectrum could be due to filtering by another pigment, but this explanation does not account for an additional, minor behavioral response to longer wavelengths near 600 nm. In contrast to the *Aq-Cry2* gene with pigment ring expression, *Aq-Cry1* did not show measurable absorption in the near-UV/visible wavelength range, possibly because it lacks flavin and therefore lacks a light response, meaning its function remains unknown (Fig. 5B). Flavins are not covalently bound to photolyases/cryptochromes, so it is not unusual to purify a protein without stoichiometric flavin (Zhao et al., 1997).

Unlike several previously studied animal Type 1 cryptochromes (Ozturk et al., 2009), we found that *Aq-Cry2* lacked photo-induced proteolysis (Fig. 5E,F). When expressed in insect S2 cells and exposed to blue light, *Drosophila* CRY was degraded after 90 min whereas *Aq-Cry2* remained intact (Fig. 5E,F). This key difference between *Aq-Cry2* and well-characterized cryptochromes such as *Drosophila* CRY indicates that, despite similar spectral properties, *Aq-Cry2* must mediate its activation through a different, as-yet-undetermined mechanism.

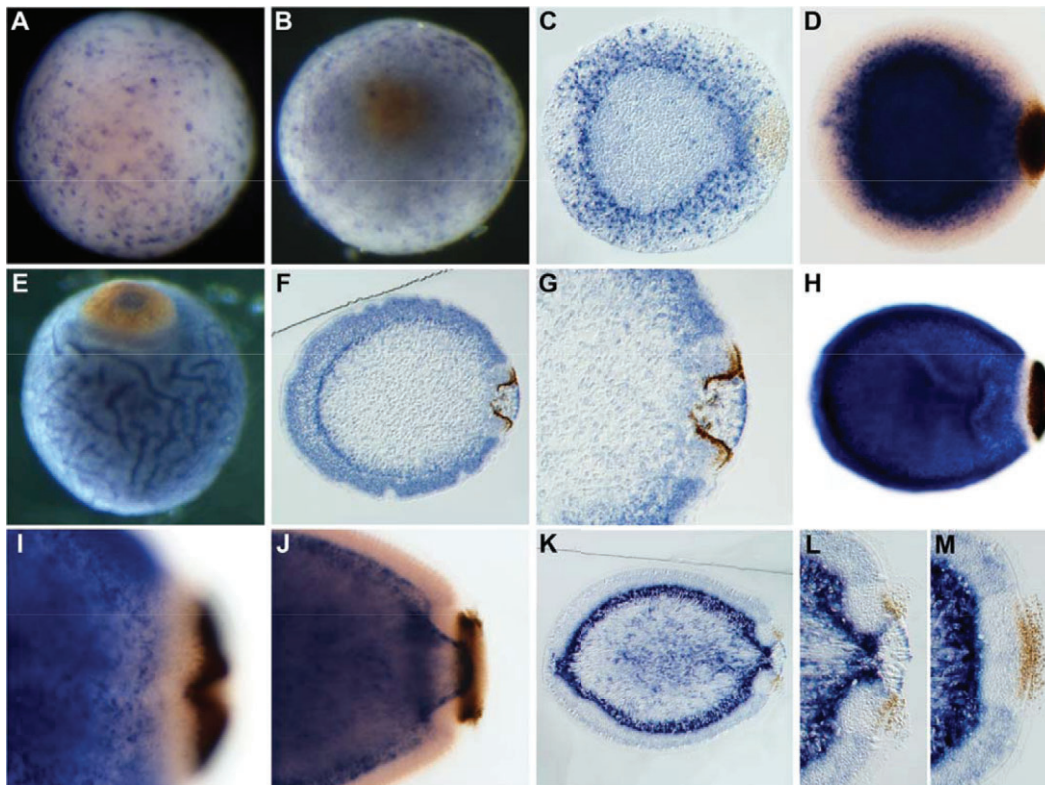


Fig. 3. Developmental expression of *Aq-Cry1*. (A,B,E) Whole-mounts; (C,F,G,K,L,M) sections; (D,H,I,J) cleared whole-mounts; (B,E) posterior pole facing up; (C,D,F–M) posterior pole to right. (A,B) In early gastrula and spot stages, *Aq-Cry1* expression is evident in maternal follicle cells on the surface of the embryo. (C,D) After gastrulation, at the spot stage, embryonic *Aq-Cry1* expression is strongest in a population of cells scattered throughout the outer region of the embryo. (E,F) During ring formation, the gene is upregulated in presumptive epithelial-like cells and in a population of cells inside the pigment ring. (G) Higher magnification of the embryo shown in F, showing expression diminished in cells adjacent to the outer part of the ring. (H) Excluding cells adjacent to the pigment ring, expression in the outer layer continues in early larvae (pre-release). (I) Higher magnification of the embryo shown in H. (J,K) In hatched, swimming larvae, *Aq-Cry1* is expressed in sub-epithelial cells and cells located inside the ring, and in the epithelial-like cells in a pattern similar to that observed at earlier stages. (L,M) Higher magnifications of the embryo shown in K, sectioned through the middle of the ring (L) or the edge of the ring (M).

## DISCUSSION

Pigment ring eyes of sponges such as *A. queenslandica* provide a fascinating case study in convergent evolution. Although all eyes of eumetazoan animals examined to date utilize phototransduction cascades initiated when photons strike homologous opsin proteins, *A. queenslandica* provides an exception to this rule (Plachetzki et al., 2007). Without neurons, and with a genome lacking opsin, this sponge's eye ring must utilize different and evolutionarily convergent molecular machinery compared with other animal eyes. An important candidate gene for involvement in these pigment ring eyes is cryptochrome (Leys et al., 2002).

Here we present correlative evidence for the function of cryptochrome in the *A. queenslandica* eye. After finding candidate genes based on sequence similarity, we showed that one of two genes (*Aq-Cry2*) lacks DNA repair activity, and is therefore a true cryptochrome, not a photolyase. We then showed that *Aq-Cry2* is expressed in the cells thought to mediate the light response (Leys and Degnan, 2001). Further, its absorption pattern shows a prominent peak corresponding to peak phototactic behaviors in *A. queenslandica* (Leys et al., 2002). This peak absorption disappears after photoreduction of *Aq-Cry2*, demonstrating photoresponsiveness of *Aq-Cry2* itself. These results are consistent with the hypothesis that opsin-less sponge eyes utilize cryptochrome, along with other proteins, to direct or act in eye-mediated phototactic behavior.

Although our expression and *in vitro* biochemical results suggest that *Aq-Cry2* functions by responding to light in the pigment ring eye, we cannot yet determine the precise nature of this function in the organism. There are multiple possibilities, including acting as the primary photoreceptor effecting ciliary rudders or acting in the entrainment of timing cues for swimming and settlement. The first possibility, *Aq-Cry2* is the primary photoreceptor, is supported by our finding that the peak sensitivity of the behavioral response is similar to the peak of the *Aq-Cry2* absorption spectrum. However, a minor behavioral response to light near 600 nm may require either a second *Aq-Cry2* co-factor (besides flavin), or a second photoresponsive protein mediating the long-wavelength response. As we see no evidence for a second co-factor in our study or in other cryptochromes in the literature that could explain a response to 600 nm light, *Aq-Cry2* may not be the primary photoreceptive molecule, or may not be acting alone.

A second possibility, that *Aq-Cry2* is involved as a timing and/or entrainment molecule, is supported by the involvement of other animal cryptochromes in entrainment (Emery et al., 1998; Stanewsky et al., 1998; Thresher et al., 1998). In this case, *Aq-Cry2* could be acting as an upstream signaling factor indicating the presence of light in general or as a developmental timing cue. As animal cryptochromes are known to function as transcription factors in signaling transduction pathways (Reppert and Weaver, 2002) as well as modifiers of protein function (Ceriani et al., 1999), *Aq-Cry2* could

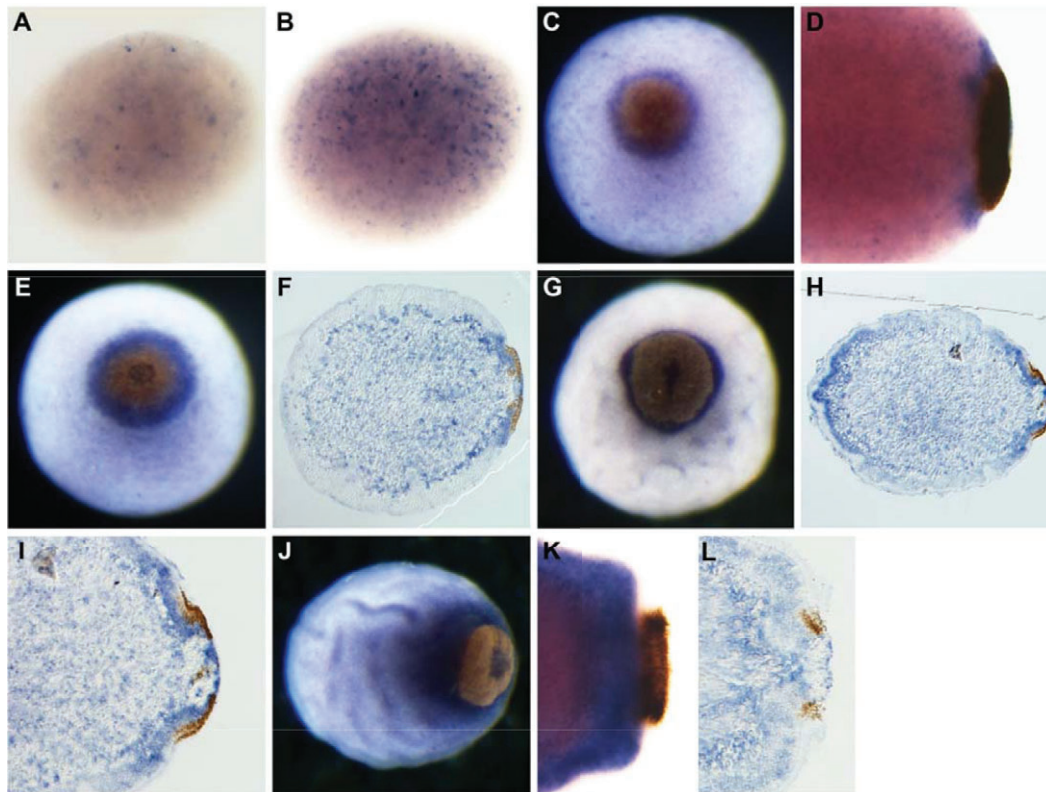


Fig. 4. Developmental expression of *Aq-Cry2*. (A,B,D,K) Cleared whole-mounts; (C,E,G,J) whole-mounts; (F,H,I,L) sections; (C,E,G) posterior pole facing up; (D,F,H–L) posterior pole to right. (A,B) *Aq-Cry2* expressing cells are scattered throughout the embryo during cleavage. (C–E) In later stages, *Aq-Cry2* transcripts are predominantly detected in the vicinity of the pigment spot and early ring. (F,G) During ring formation, *Aq-Cry2* expression continues outside, underneath and inside the ring, and also becomes evident in a population of cells located beneath the outer-layer, putative sub-epithelial cells. (H–J) As the embryo develops into an early larva, expression of the gene increases in the outer layer, appearing strongest at the anterior and posterior poles. (I) Higher magnification of the embryo shown in H. (K,L) In swimming larvae, *Aq-Cry2* is expressed in sub-epithelial cells, epithelial-like cells, pigment cells, cells with long cilia and in a subset of cells located inside the pigment ring.

be activating, *via* transcription or protein modification, other downstream factor(s) responsible for the light response. If *Aq-Cry2* is indeed acting as a cue, rather than an effector, then it only needs to be tuned to the major wavelengths of light associated with the behavior (420–460 nm). Future studies into genes involved in entrainment could give insight into the function of sponge cryptochromes. For example, if other known entrainment molecules are expressed in the developing pigment ring, then a role for *Aq-Cry2* in entrainment could be supported. If so, a different and unknown photoreceptive molecule could be the directional indicator needed to guide swimming behavior.

Other candidate genes for the primary photoreceptive molecule, beside cryptochrome, are also worthy of consideration. The short-wavelength receptor *lite-1* was recently characterized in *Caenorhabditis elegans* (Edwards et al., 2008) and *D. melanogaster* (Xiang et al., 2010). However, *lite-1*, like opsin, acts in neurons and would therefore require a different mechanism in sponges. Another candidate is the mitochondrial gene cytochrome oxidase I (CO1). The absorption spectrum for CO1 closely matches the entire behavioral action spectrum for *A. queenslandica*, including the minor long-wavelength behavioral response (Bjorn and Rasmusson, 2009). Although CO1 is not generally thought of as a light-responding molecule, it has been shown to absorb light in various biological contexts (Hallén and Brzezinski, 1994; Karu, 1999; Karu et al., 2004). However, a

mechanism for how CO1 could mediate ciliary responses is unknown. An additional difficulty is that testing whether CO1 functions as a photoreceptor is challenging. A search of the *A. queenslandica* genome did not uncover a nuclear copy of CO1, and expression studies of a mitochondrial gene would be uninformative, in part because increased concentration could represent increased numbers of mitochondria for energetic needs that may be required by active ciliated cells, rather than indicating localization of a photoreceptive gene. Furthermore, knockdown of a mitochondrial gene such as CO1 would cause numerous side effects, and preclude testing its function as a photoreceptor.

Given our expression and biochemical results, elucidation of the exact function of *A. queenslandica* cryptochromes provides rich prospects for future research that will depend on multiple complementary approaches. Although a gene knockdown approach (Rivera et al., 2011) could confirm that *Aq-Cry2* plays some role in larval phototaxis, it may not be able to distinguish between a specific role as the photoreceptor regulating the ciliary rudder, an ongoing role in settlement timing, or both. Indeed, both duplicate sponge cryptochromes are expressed throughout development and it would not be surprising if they had multiple functions at different developmental time points. Another hurdle is that techniques for gene knockdown in sponges are limited to feeding adults and have not yet been developed for embryos or larvae, the stage when the animals are phototactic (Rivera et al., 2011). In light of this, experiments

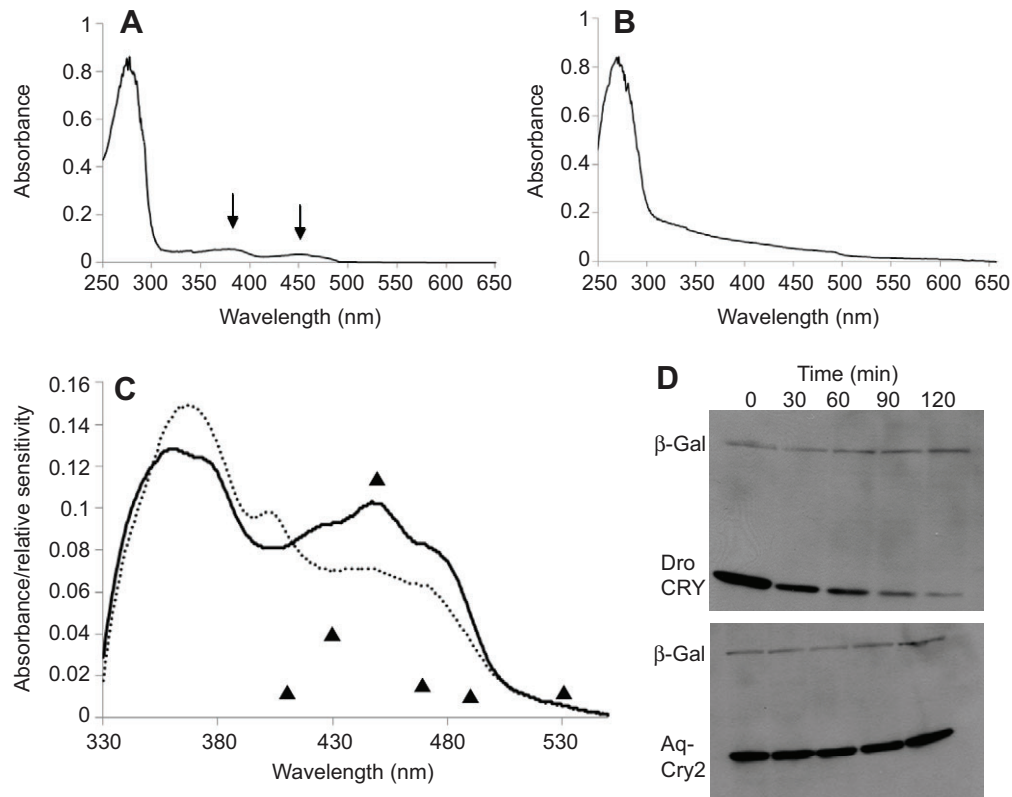


Fig. 5. Spectroscopic properties and proteolytic activity of *A. queenslandica* cryptochromes. (A,B) Absorption spectra of purified *Aq-Cry2* (A) and *Aq-Cry1* (B). Arrows in A point to peaks, indicating purification of the protein along with stoichiometric flavin. The lack of measurable absorption in the 350–350 nm range in B suggests that *Aq-Cry1* (AqCRY1-V-5/HisA) did not contain substantial flavin after purification. MBP-AqCry1 showed the same result (data not shown). In contrast, the ratio of absorbance at 280 nm of apoprotein to the 450 nm flavin adenine dinucleotide absorption peaks suggests that the recombinant MBP-AqCry2 contained stoichiometric flavin. (C) Photoreduction of *Aq-Cry2* by blue light. Solid line: spectrum of *Aq-Cry2* purified under yellow light. Dashed line: after exposure to UV light (366 nm at  $2\text{ mW cm}^{-2}$  for 15 min). *Aq-Cry2* showed the typical type-1 cryptochrome reduction pattern, with an increase of the 366 nm peak and the formation of a new peak at 405 nm and concomitant decrease of the 450 nm peak. Triangles indicate the behavioral response in living larvae (Leys et al., 2002); the minor behavioral response to 600 nm light not shown (units for behavioral response are arbitrary). (D) Light-induced proteolysis of cryptochromes. *Drosophila* CRY (Dro CRY) and *Aq-Cry2* were transfected into S2 cells alongside with beta-galactosidase ( $\beta$ -Gal) as an internal control, and tested for light-induced proteolysis after 36 h. Although the *Drosophila* CRY protein was degraded via light-induced proteolysis (upper panel), levels of *Aq-Cry2* remained high even after 2 h of light activation (lower panel).

manipulating magnetic fields (Henbest et al., 2008) or examining a potential role for cryptochromes in the regulation of ciliary movements via calcium signaling (Liscum et al., 2003; Tamm and Terasaki, 1994) might be used to provide mechanistic insight into *Aq-Cry2* function.

This study explored the molecular basis of sponge vision, which likely evolved in the absence of neurons and opsins. Although eyes have evolved up to 60 times in eumetazoan animals (Salvini-Plawen and Mayr, 1977), all of these share many genetic components (Kozmik, 2008; Mutsuddi et al., 2005). In particular, they are opsin-based (Spudich et al., 2000; Suga et al., 2008; Vopalensky and Kozmik, 2009), illustrating an evolutionary continuity that runs through all non-poriferan animal eyes examined to date. Of particular interest for future study will be to elucidate multiple molecular components of sponge phototaxis, and to determine whether any are shared with non-poriferan eyes, or whether the sponge eyes are instead completely convergent at the genetic level.

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