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Identification and Characterization of Cryptochrome4 in the Ovary of Western Clawed Frog *Xenopus tropicalis*

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CRY proteins can be classified into several groups based on their phylogenetic relationships, and they function as a photoreceptor, a photolyase, and/or a transcriptional repressor of the circadian clock. In order to elucidate the expression profile and functional diversity of CRYs in vertebrates, we focused on XtCRY4, a member of the uncharacterized cryptochrome family CRY4 in *Xenopus tropicalis*. XtCRY4 cDNA was isolated by RT-PCR, and a phylogenetic analysis of deduced sequence of XtCRY4 suggested that the vertebrate *Cry4* genes evolved at much higher evolutionary rates than mammalian-type *Cry* genes, such as the CRY1 and CRY2 circadian clock molecules. A transcriptional assay was performed to examine the transcriptional regulatory function as circadian repressor, and XtCRY4 had marginal effects on the transactivation of XtCLOCK/XtBMAL1 via E-box element. In situ hybridization and quantitative RT-PCR was performed to detect mRNA expression in native tissues. Quantitative RT-PCR revealed that *XtCry4* mRNA was highly transcribed in the ovary. In situ hybridization showed the presence of *XtCry4* transcripts in the oocytes, testis, renal tubules, the visual photoreceptors, and the retinal ganglion cells. A specific antiserum to XtCRY4 was developed to detect endogenous expression of XtCRY4 protein in the ovary. The expression level was estimated by immunoblot analysis, and this is the first detection and estimation of endogenous expression of CRY protein in the ovary. These results suggest that *X. tropicalis* ovary may respond to blue-light by using XtCRY4.

Key words: cryptochrome, circadian clock, clock-related molecule, ovary, oocyte, photoreceptor, testis

INTRODUCTION

Cryptochromes (CRYs) are flavoproteins found in a wide range of organisms, including bacteria, plants, fungi and animals (Hsu et al., 1996). While CRYs exhibit high homology with photolyases, which receive blue light to repair UV-damaged DNA (Todo et al., 1996), most CRYs lack such photorepair activity (Kobayashi et al., 2000). *Drosophila* CRY has been shown to operate as a blue-light photoreceptor capable of resetting the circadian clock (Emery et al., 1998; Stanewsky et al., 1998) and has recently been implicated in magnetoreception (Gegear et al., 2008).

Three groups of CRY genes, *Cry1*, *Cry2*, and *Cry4*, have been identified in non-mammalian vertebrate species. In most, if not all, classes of vertebrates, CRY1 and CRY2 proteins are thought to function as core transcriptional repressors in the transcription/translation-based negative feedback mechanism of the circadian clock (Griffin et al., 1999; King and Takahashi, 2000; Kume et al., 1999; Yamamoto et al., 2001; Zhu and Green, 2001; Kubo et al., 2010). That is, CRY1 and CRY2 negatively control the transcription of core clock genes such as *Pers* through the

CACGTG-type E-box, a core circadian enhancer element by inhibiting CLOCK and BMAL-containing transactivator complex. In addition, CRY1 and CRY2 likely operate not only as circadian clock components, but also as photoreceptors in non-circadian functions, at least in some avian and fish species (Mouritsen et al., 2004; Tu et al., 2004; Fukushima et al., 2011; Kubo et al., 2010). We previously reported that the mRNA level of SgCRY3, a close relative of CRY2, in the medial brain of the lunar-responding fish, goldlined spinefoot (*Siganus guttatus*), fluctuates in accordance with lunar cycle, implicating SgCRY3 as a state-variable in the putative lunar estrus cycle (Fukushima et al., 2011). Thus, CRYs may contribute to the circadian clock and a wide range of photic and chronobiological physiologies in vertebrates.

The physiological function of CRY4 is unidentified: chicken CRY4 (cCRY4) and zebrafish CRY4 (zCRY4) show no inhibitory effect on CLOCK/BMAL1-driven transactivation via the E-box sequence (Kobayashi et al., 2000; Kubo et al., 2006). Though recent findings in which we detected a light-dependent structural change of cCRY4 in a retinal soluble fraction (Watari et al., 2012) suggest a photoreceptive role (i.e., circadian photoreception or light-dependent magnetoreception) of CRY4 in the retina, we have very little information about the tissue distribution and physiological function of CRY4 in other animals.

At the initial stage of this study, we searched public genome databases and found *Cry4* orthologs in the frog (*Xenopus laevis*, *Xenopus tropicalis*), green anole (*Anolis carolinensis*), and amphioxus (*Branchiostoma floridae*). The

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western clawed frog, *Xenopus tropicalis* (*X. tropicalis*), makes an excellent lower vertebrate animal model and became our focus in this study because of the following experimental advantages: (1) its entire genomic has been sequenced, (2) smaller genome size (1.7 Gbp) compared to its close relative *X. laevis* (3.1 Gbp) (Thiebaud and Fischberg, 1977), and (3) more rapid maturation (within six months) than *X. laevis* (approx. 1 year) (Hirsch et al., 2002).

In this study, we cloned an entire coding sequence of XtCRY4, examined its transcriptional regulatory function by luciferase assay, and investigated *XtCry4* mRNA expression using qRT-PCR and in situ hybridization. Of the examined tissues, *XtCry4* mRNA level was extremely high in the ovary. Because of a lack of information on CRY protein expression in the ovary of any animals to date, we tried to detect XtCRY4 protein expression by preparing a specific antibody. Results of these analyses indicate the expression of XtCRY4 in the *X. tropicalis* ovary and imply considerable diversity of function and expression of *Cry* family molecules among vertebrate species.

MATERIALS AND METHODS

Animals

All animals were treated in accordance with the guidelines of WASEDA University (permission # 08A03, 09A02, 10A02, WD10-91, WD11-84, 09A04, 10A04). *X. tropicalis* (Yasuda line) was kindly provided by Dr. Takase and Dr. Yaoita (the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT); Institute for Amphibian Biology, Graduate school of Science, Hiroshima University), or bred from these frogs. Adult *X. tropicalis* were entrained in 12 h-light / 12 h-dark (LD) cycles for at least two weeks before the initiation of experiments.

Cloning of cDNA of *XtCry4* encoding full-length coding sequences

Total RNA was extracted from *X. tropicalis* larvae using TRIzol reagent (Life Technologies), and first-strand cDNAs were synthesized with SuperScript III reverse transcriptase (Life Technologies) using oligo(dT)₂₁ primer. Primers for cDNA cloning were designed based on database-deposited partial sequences, which anneal to the untranslated region of *XtCry4* gene (5'-CACCG GAGAC TTGAA ATTCA GAAGC-3' and 5'-ATATC TGAAA AGGCC CCAGT TC-3'). PCR products amplified with *PfuUltra* (Agilent Technologies) were inserted into the pMD20-T vector (TaKaRa, Japan), and the resulting vector was named pMD20-T-XtCry4. The inserts of at least three independent clones were sequenced. Full-length cDNAs without presumed PCR errors were isolated.

Transcriptional assay

Using pMD20-T-XtCry4 as a template, *XtCry4* cDNA was amplified with primers (5'-CACCG ACCTG CCCAC CATGC CTCAT CGGAC AATCC AC-3' and 5'-GCTCT TCTCA GCACA CTTTC ACTTC ACAAG G-3'; codons for translation initiation and termination are underlined) and subcloned into the pENTR/D-TOPO vector (Life Technologies). An entry vector without PCR error (termed pENTR/D-XtCRY4) was isolated, and internal *XtCry4* cDNA was transferred into the pcDNA-3.2/V5-DEST mammalian expression vector (termed pcDNA-3.2-XtCry4) using LR clonase (Life Technologies). The resultant expression vector and vectors for expression of XtCLOCK, XtBMAL1, XtCRY1, and XtCRY2 and the luciferase reporter vector *XtPer1 E-box-SV40-luc*, formerly termed *XtPer1-luc*, were transfected into human embryonic kidney (HEK) 293 cells (RIKEN CELL BANK, Japan) and harvested as described previously (Kubo et al., 2010). Transcriptional assays were performed using

the Dual-Luciferase Reporter Assay Kit (Promega).

Cellular localization of CRY4 in HEK 293 cells

XtCRY4 cDNA was transferred from the pENTR/D-XtCRY4 vector to pcDNA-DEST53 vector (Life Technologies) by using LR clonase (Life Technologies) to construct a mammalian expression vector (termed pcDNA-GFP-XtCRY4) encoding a GFP-XtCRY4 fusion protein. pcDNA-GFP-XtCRY1 or pcDNA-GFP-XtCRY2 (Kubo et al., 2010) or pcDNA-GFP-XtCRY4 or pcDNA-DEST53-empty was transfected to HEK 293 cells using Lipofectamine (Life technologies) and Plus Reagent according to the manufacturer's instructions.

Quantitative RT-PCR analysis

X. tropicalis tissues were collected at Zeitgeber time 6 (ZT6) under fluorescent light (~300 μW/cm²) or at ZT18 under dim red light (> 640 nm, ~120 μW/cm²), and kept in RNAlater (Ambion) at 4°C until RNA extraction. Total RNA was extracted from the tissues using TRIzol reagent (Life Technologies). Quantitative RT-PCR analyses of 1 μg of total RNA were performed using StepOnePlus (Applied Biosystems) along with the High Capacity cDNA Reverse Transcription Kit and Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences for qRT-PCR are shown in Table 1. As internal expression control genes, we used *XtHprt* for expression profiles among tissues except for testis, because of their stable expression across tissues except for testis, and we used the geometric mean of *XtHprt1* and *Xtβ2M* for temporal variation because of their relatively constant expression over the span of a day (Kubo et al., 2010).

In situ hybridization

A 437 bp sequence of the *XtCry4* gene (nucleotides 1471–1671 of the open reading frame (ORF) and downstream 236 bp in the 3'-UTR) was amplified with PCR using the following primers: for antisense probe, 5'-TAATA CGACT CACTA TAGGG ATGGT GCCA GGTAC G-3' and 5'-GGATA TCCAA AAGAC TGCAC AACTT ACC-3'; for sense probe, 5'-TAATA CGACT CACTA TAGGG CAAAA GACTG CACAA CTTAC C-3' and 5'-GGATA TCGAT GGTGC CCAGG TACG-3'. Probes for *rhodopsin* were prepared by PCR amplification of a 500 bp sequence of the *X. tropicalis rhodopsin* gene (nucleotides 1024–1523 of the C-terminal alanine and the downstream 497 bp in the 3'-UTR) using the following primers: for antisense probe, 5'-TAATA CGACT CACTA TAGGG CAGGA TTTTA ATGCA AGGGG TCTGG C-3' and 5'-GGATA TCGCA TAAGA GCTTC ACCCA AGGCT G-3'; for sense probe, 5'-TAATA CGACT CACTA TAGGG CATAA GAGCT TCACC CAAGG CTG -3' and 5'-GGATA TCGCA GGATT TTAAT GCAAG GGGTC TGGC -3'. The resulting PCR fragments were cloned into the pMD20-T vector and used for preparing digoxigenin-labeled RNA probes with the aid of T7 RNA polymerase (TaKaRa, Japan) and DIG RNA Labeling Kit (Roche). Hybridization of the RNA probe in this section

Table 1. List of primers for quantitative RT-PCR.

Gene	Primer	Sequences
<i>XtCry1</i>	XtCry1-taq-F	5'-TGGCGTGCTTCCTCACCA-3'
	XtCry1-taq-R	5'-CCTGCATTACGCTCCAATCA-3'
<i>XtCry2</i>	XtCry2-taq-F	5'-TGTTTCGCTTTGGAAGGCGC-3'
	XtCry2-taq-R	5'-CTATGTCCGTTTCAGCTCGATTATC-3'
<i>XtCry4</i>	XCry4-F	5'-TGTTTCGCTTTGGAAGGCGC-3'
	XCry4-R	5'-CCACCATAGGAAGAGGATAATCTT-3'
<i>Xtβ2M</i>	XtB2M-taq-F	5'-GTGCACATCGACAGCGATG-3'
	XtB2M-taq-R	5'-GTCTGCGGCTCAGAACATG-3'
<i>XtHprt1</i>	Xhprt-taq-F	5'-AGGCTCAGACATGGCGAG-3'
	Xhprt-taq-R	5'-GTGGAATGTAGACTTTCTCCAGATC-3'

was carried out as previously described (Watari et al., 2012) plus the tissue section on the slide glass was pre-treated with Proteinase K solution (2.5 µg/ml Proteinase K, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) for 10 min at 37°C. The hybridized probe was detected using the DIG Nucleic Acid Detection Kit (Roche).

Production of polyclonal antibody to XtCRY4

Two kinds of fusion proteins were prepared: GST-XtCRY4CCT is composed of glutathione S-transferase (GST) and the cryptochrome carboxyl-terminal region (CCT) of XtCRY4 protein (Asp⁴⁷¹-Cys⁵⁵⁷; XtCRY4CCT), and MBP-XtCRY4CCT is composed of maltose-binding protein (MBP) and XtCRY4CCT. Two kinds of fusion proteins were expressed in *Escherichia coli* BL21, purified as described previously (Okano and Fukada, 2000). MBP-XtCRY4CCT and GST-XtCRY4CCT were used as antigens for immunization and immunoaffinity purification, respectively.

Immunoblot analysis

X. tropicalis ovary was homogenized using a Potter-type Teflon homogenizer with PBS [10 mM NaH₂PO₄, 140 mM NaCl, 1 mM MgCl₂, pH 7.4]. As a positive control, XtCRY4 was exogenously expressed in HEK 293 cells. pcDNA-DEST53-XtCRY4 or pcDNA-DEST53-empty were transfected to HEK 293 cells using Lipofectamine (Life technologies) and Plus Reagent according to the manufacturer's instructions. Fifty-two hours after the transfection, each cell was washed and harvested with PBS. These tissue and cellular samples were subjected to SDS-polyacrylamide (10%) gel electrophoresis, followed by electroblotting onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membrane was incubated for 1 h in 4% Block Ace (Dainippon Pharmaceutical, Japan), and then again with the primary antibody (anti-XtCRY4CCT antibody or anti-GFP (#012-22541, Wako) or control IgG (#I5381, Sigma)) diluted in 0.4% Block Ace for 24 h at 4°C. Subsequently, the membrane was washed with 0.4% Block Ace and incubated with an alkaline phosphatase-linked anti-mouse IgG antibody (1 : 1000 dilution, New England Biolabs) in 0.4% Block Ace for 24 h. Signals were detected using CDP-Star Reagent (New England Biolabs).

Statistical analysis

Data were analyzed using the Student's t-test or ANOVA with Tukey-Kramer multiple comparisons on Statcel2 (the add-in forms on Microsoft Excel) software.

RESULTS

cDNA cloning and phylogenetic analysis of XtCRY4

Based on the sequence information found in the Ensembl and EST database, we designed a primer set to amplify *XtCry4* cDNA that would cover the entire coding sequence from *X. tropicalis* larvae cDNA, and then determined all coding sequences without PCR errors (Fig. 1, Genbank acc. No. **AB534559**). In comparing the cDNA sequence with the genome database (Ensembl JGI_4.2 and

Xenbase 7.1), we noticed that the *XtCry4* gene was more than 20 kbp long and consisted of 11 exons. A noticeably long intron was located 19 bp upstream of the initiation AUG codon (GGAGACTTGAA [gt ...7861 bp... ag] ATCA-GAAGCTGCTCCACCATG).

We performed a phylogenetic analysis of the CRY/photolyase family proteins using the amino acid sequence from XtCRY4 and its putative orthologous sequence found in *Xenopus laevis* (Fig. 2). In this phylogenetic tree, XtCRY4 belongs to the vertebrate CRY4 group and forms a monophyletic group, which clearly diverges from the mammalian CRY1/2 group.

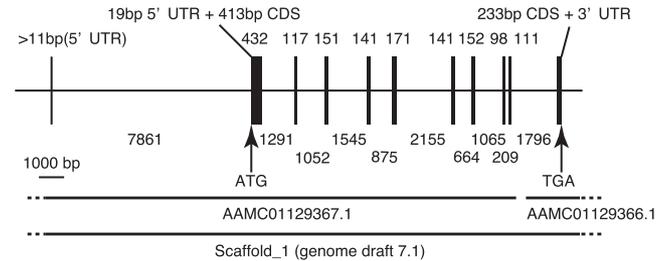


Fig. 1. Genomic organization of *XtCry4* gene. The *XtCry4* cDNA sequence was aligned with the genome database (Ensembl JGI_4.2 and Xenbase 7.1) as of 25 March 2013, and exon/intron boundaries were assigned to fulfill GT-AG rule. Exons are indicated by vertical lines. The lengths of exons and introns are shown above and below the genomic structure, respectively.

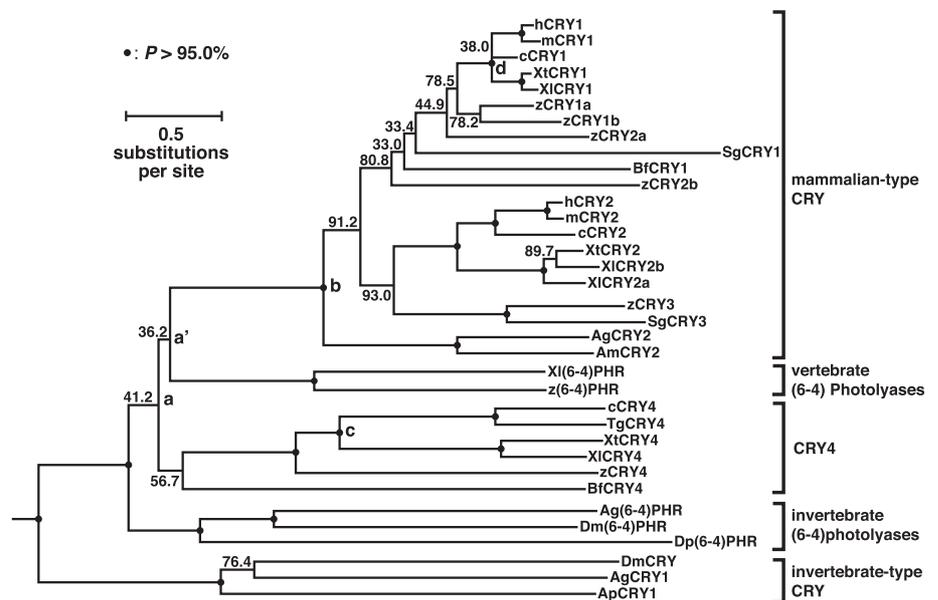


Fig. 2. A phylogenetic tree of CRY family proteins. XtCRY4 sequence and related protein sequences were obtained from the NCBI Entrez Protein database (accession nos. are shown in Table S1). These were analyzed in the conserved region of the CRY family proteins (corresponding to Ile⁶-Pro⁴⁶⁶ in XtCRY4) using the Neighbor-Joining method and CLUSTAL W and NJ plot software (version 2.3). CRY DASH proteins (XICRY DASH, zCRY DASH, and AtCRY DASH) were used as the outgroup (not shown). Bootstrap probabilities (*P*) are represented by closed circles on the nodes (*P* > 95%) or values near the nodes. Abbreviations are defined as follows: h, human; m, mouse; c, chicken; Xt, *Xenopus tropicalis*; XI, *Xenopus laevis*; z, zebrafish; Sg, *Siganus guttatus*; Bf, *Branchiostoma floridae*; Tg, *Taeniopygia guttata*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Ap, *Antheraea pernyi*; Dm, *Drosophila melanogaster*; Dp, *Danaus plexippus*; PHR, photolyase. For letter symbols (a, a', b, c, d), see text.

Transcriptional analysis and cellular localization of XtCry4

To assess the function of XtCRY4 as a transcriptional repressor, we performed a luciferase assay using HEK 293 cells to test whether XtCRY4 has the ability to repress CLOCK/BMAL-dependent transactivation from the E-box element (Fig. 3). XtCRY4 showed no inhibitory effect on the transactivation of CLOCK/BMAL1 from the E-box sequence,

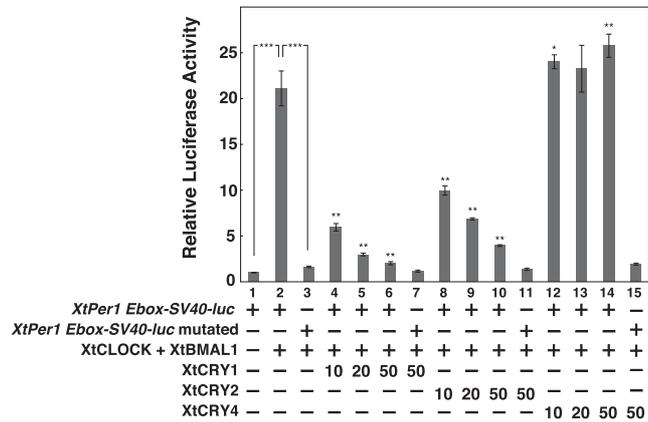


Fig. 3. Effects of XtCRY on XtCLOCK:XtBMAL1-mediated transactivation from *XtPer1* E-box elements. We mixed 125 ng of a firefly luciferase reporter with *XtPer1 E-box-SV40-luc* reporter, 500 pg of *Renilla* luciferase reporter, pRL-TK as an internal control (Promega), 250 ng of XtCLOCK expression vector, 25 ng of XtBMAL1 expression vector, and 0, 10, 20, or 50 ng of XtCRY expression vector. The total amount of plasmids was adjusted to 1 µg per well of 6-well plate by adding pcDNA/V5-DEST empty vector. All data presented are the means ± SE for three independent experiments. ****P* < .0001, Student's t-test, **P* < 0.05, ***P* < 0.01, Tukey-Kramer test, comparing the effect of 4, 5, 6 to 2; or 8, 9, 10 to 2; or 12, 13, 14 to 2.

while both XtCRY1 and XtCRY2 markedly inhibited the transactivation as previously reported (Kubo et al., 2010). We examined the cellular localization of XtCRY4 by investigating the localization of XtCRY4 GFP-fusion protein (GFP-XtCRY4) exogenously expressed in HEK 293 cells. Consistent with a lack of transcriptional repressor activity, GFP-XtCRY4 was mostly observed in the cytoplasm (Fig. 4).

Messenger RNA analysis of XtCry4 by quantitative RT-PCR

We next collected twelve tissues (*n* = 4) from adult *X. tropicalis* at midday (ZT6) or midnight (ZT18) and investigated the expression levels of *XtCry4* mRNA by qRT-PCR analysis (Figs. 5–7). Because threshold cycles for *Xtβ2M* in the qRT-PCR analysis were similar among the tissues, except for testis in which *Xtβ2M* was not significantly detected, we evaluated expression levels in tissues other than testis by averaging those values for ZT6 or ZT18 (Fig. 5). *XtCry4* expression levels were extremely high in the ovary and the levels were approximately 40 times higher than those in the kidney, the next most abundant tissue, and 6330 times more than those in the retina, the least abundant tissue examined in the present experiment. When comparing the mRNA levels at ZT6 and ZT18, the mRNA levels of *XtCry4* did not show significant differences in most tissues examined but showed weak variation in the brain, heart, and stomach (Fig. 6). Because threshold cycles for *XtHprt1* was similar between ovary and testis, we compared mRNA levels of *XtCrys* in the testis with those in the ovary by using *XtHprt1* as the reference gene. We found relatively high levels of *XtCrys* expression (Fig. 7).

In situ hybridization

We performed in situ hybridization for further detection of the cells expressing *XtCry4* mRNA (Figs. 8 and 9). In the ovary, we found a strong signal for *XtCry4* mRNA observed in the cytoplasm (yolk granules, YG in Fig. 8C) of oocytes. Granulosa cells are not clearly identified, but theca cells at the later stages are likely negative (Fig. 8A; TC in Fig. 8C). In the testis, Leydig cells (LC in Fig. 8G) are positive, and additionally positive cells (Sertori cells and/or spermatocytes) were observed in the seminiferous tubules (Fig. 8E). In the kidney, relatively intense signals were observed in renal tubule cells (Fig. 9A). Although qRT-PCR analysis (Fig. 5) enabled us to identify the lowest level of *XtCry4* mRNA in the retina, the mRNA expression was further localized using in situ hybridization (Fig. 9C), since *Cry4* mRNA level is known to be highly transcribed in the chick retina (Kubo et al., 2006). In situ hybridization with longer incubation in the detection process resulted in intense signals for *XtCry4* mRNA at the outer nuclear layer (ONL), inner nuclear layer (INL) and in cells of the

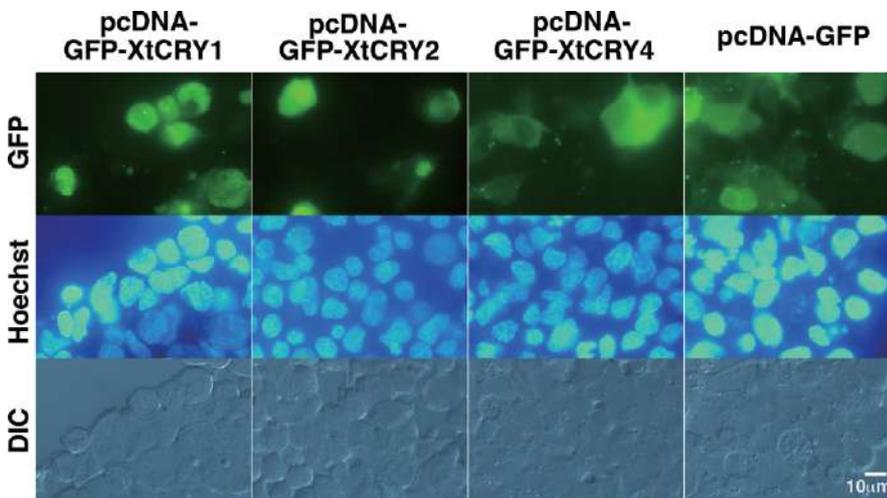


Fig. 4. Cellular localization of GFP-fused XtCRY4 expressed in HEK 293 cells. Expression vector (pcDNA-GFP-XtCRY1 or pcDNA-GFP-XtCRY2 or pcDNA-GFP-XtCRY4 or pcDNA-GFP) was transfected into HEK 293 cells. After 72 h, the cells were treated Hoechst 33342 (Dojindo), and observed using a differential interference microscope (lower, DIC) or a fluorescence microscope for detection of GFP (upper, GFP) and nuclei (middle, Hoechst). GFP-XtCRY1 and GFP-XtCRY2 were observed in the nucleus as reported previously (Kubo et al., 2010).

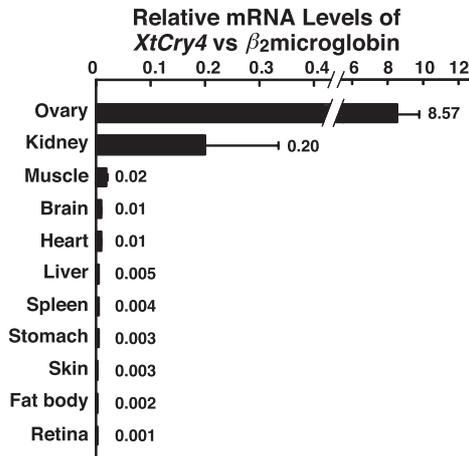


Fig. 5. Tissue specificity of *XtCry4* mRNA levels. Messenger RNA levels at ZT6 and ZT18 are averaged and shown as ratios to *Xtβ2M* mRNA levels.

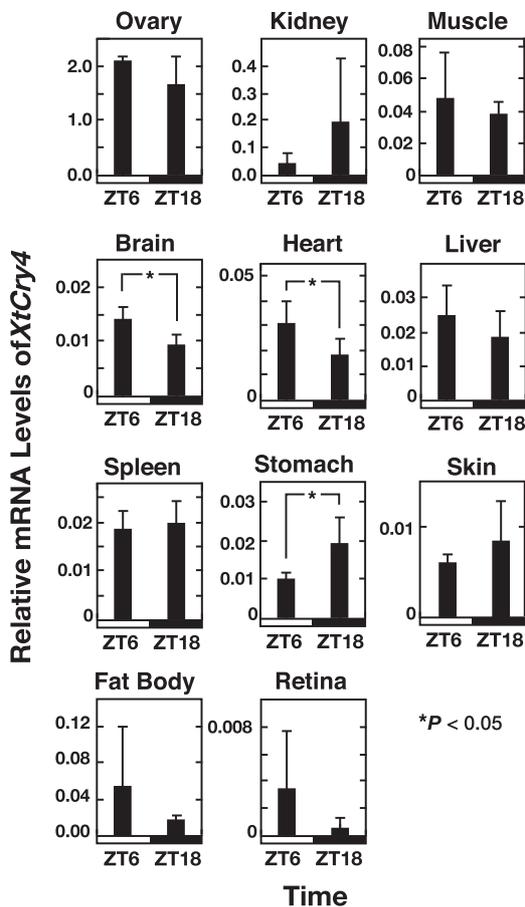


Fig. 6. Day-night expression levels of the *XtCry4* mRNA levels in eleven tissues. Messenger RNA levels are shown as a ratio to geometric mean of *XtHprt1* and *Xtβ2M* mRNA levels. * $P < 0.05$, Student's t-test.

retinal ganglion cell layer (GCL) (Fig. 9C). Judging from the outer segment structures, *XtCry4*-positive cells in ONL were identified to be rods (Fig. 9C), which are also labeled with *Rhodopsin*-probe (Fig. 9E).

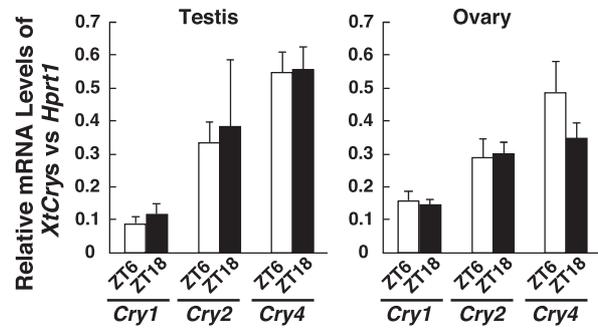


Fig. 7. Day-night expression levels of the *XtCry1*, *XtCry2*, and *XtCry4* mRNA levels in the testis and ovary. Messenger RNA levels are shown as a ratio to *XtHprt1* mRNA levels.

Western blot analysis

The extremely high expression of *XtCry4* transcript seen in the ovary (Fig. 5) led to further detection of the XtCRY4 protein and production of the anti-XtCRY4 antibody. Because cryptochromes are known to have diverging amino acid sequences in the CCT region (Watari et al., 2012), the GST-XtCRY4CCT fusion protein was used as the antigen and the antiserum was purified by immunoaffinity column chromatography using MBP-XtCRY4CCT-immobilized gel. Specificity of the purified antibody was tested by immunoblot analysis against fusion proteins of GFP and XtCRY proteins (Fig. 10, lanes 1–9). The anti-XtCRY4CCT and anti-GFP antibodies reacted with GFP-XtCRY4 recombinant protein expressed in the HEK 293 cells (weak and strong bands indicated by arrowheads in Fig. 10, lanes 3 and 6) but did not crossreact with GFP-XtCRY1 or GFP-XtCRY2 proteins (lanes 4 and 5). Duplication of the bands for GFP-XtCRY4 was likely due to the partial decomposition or post-translational modification that occurred in HEK 293 cells. Immunoblot analysis of the ovary homogenate showed a single band at 58.5 kDa (an open arrowhead in Fig. 10, lane 10), which is consistent with the predicted molecular size of XtCRY4 (64.6 kDa). Comparing the signal intensity to those with decreasing amounts of positive control protein GST-XtCRY4CCT (data not shown), the protein level of XtCRY4 in the ovary was roughly estimated as 20 ng protein/g tissue (wet weight).

DISCUSSION

A phylogenetic tree of CRY/photolyase family proteins (Fig. 2) suggests that XtCRY4, along with other vertebrate CRY4 proteins, which forms a monophyletic group that is distinct from invertebrate-type CRY, (6-4)photolyase, and mammalian-type CRY groups (vertebrate CRY1/2, fish CRY3, and insect CRY2). Vertebrate and invertebrate (6-4)photolyases form independent clusters, but relationships among these (6-4)photolyases and mammalian-type CRYs and CRY4s are relatively obscure, as indicated by lower bootstrap values (36–57%). It is clear, however, that a common ancestor of the mammalian-type CRYs has diverged from possibly a gene duplication of a common ancestor of the CRY4 group (Fig. 2, node a) or a common ancestor of the vertebrate (6-4)photolyases group (Fig. 2, node a'). This divergence likely occurred before the divergence of arthropods and vertebrates (Fig. 2, node b). A functional analysis of mammalian-type CRY in the monarch butterfly clearly

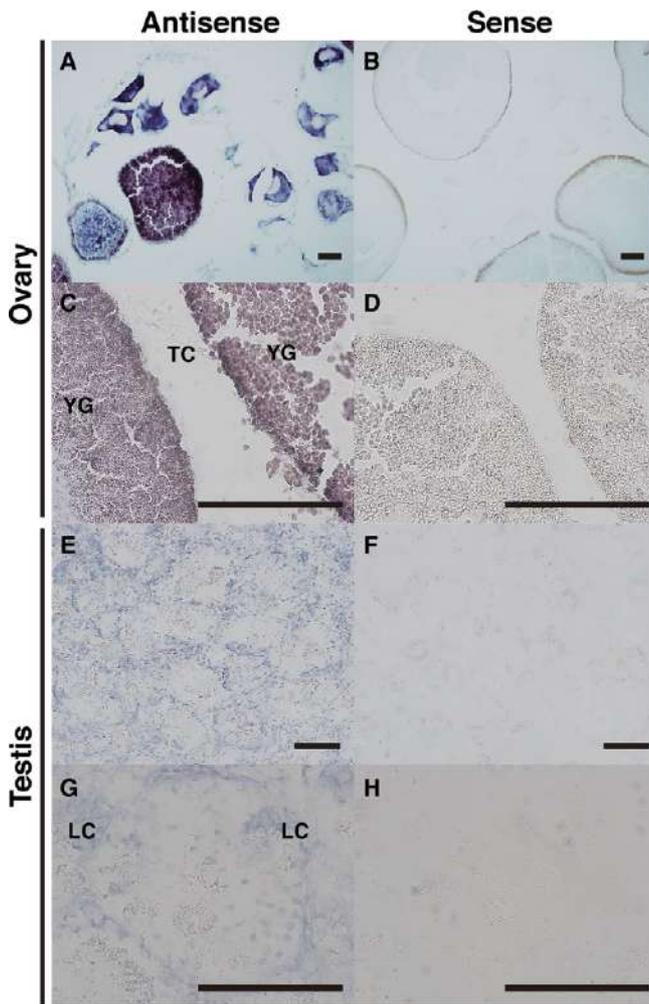


Fig. 8. In situ hybridization analysis of *XtCry4* mRNA expression in the *X. tropicalis* ovary and testis. *X. tropicalis* ovary and testis were fixed at ZT6, sectioned 14 μ m cryosection. In situ hybridization analysis was done with digoxigenin-labeled antisense (A, C, E, G) and sense (B, D, F, H) *XtCry4* probes. (A, B): ovary; (C, D): ovary (magnified view of oocytes); (E, F): testis; (G, H): testis (magnified view of seminiferous tubule). Scale bars, 100 μ m. In situ hybridization were performed following conditions; probe hybridization: 10–11 h, incubation of anti-digoxigenin antibody: 25°C, 2 h, coloration of NBT-BCIP: 9 h (ovary) or 4 h (testis).

shows an essential role for CRY2 in invertebrate circadian clock oscillation (Yuan et al., 2007; Merlin et al., 2013). Overall, it is likely that the circadian repressor function of mammalian-type CRYs had been established before the separation between vertebrates and insects (Fig. 2, node b) and that the ancestral mammalian-type CRY gene evolved from a common ancestor of CRY4 and/or (6-4)photolyases.

Comparison of the *XtCRY1* and *XtCRY4* branch lengths to divergences of amphibians and the common ancestors of higher vertebrates (Fig. 2, node c and d; lengths between node c-*XtCRY4* and node d-*XtCRY1* are 1.376 and 0.206, respectively) lead us to infer that the evolutionary rate may be much higher (approx. 6.7 times) in *CRY4* than mammalian-type CRY (*CRY1*). This difference likely reflects the stronger selection pressure in mammalian-type CRYs ver-

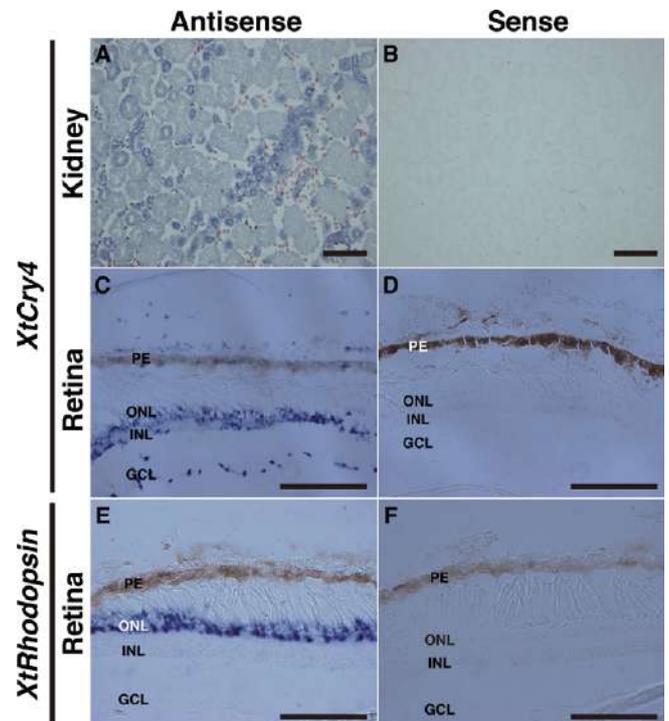


Fig. 9. In situ hybridization analysis of *XtCry4* mRNA expression in the *X. tropicalis* kidney and retina. *X. tropicalis* tissues were fixed at ZT6, sectioned 14 μ m cryosection. In situ hybridization analysis was done with digoxigenin-labeled antisense (A, C) and sense (B, D) *XtCry4* probes. Retinal sections as positive controls were hybridized with digoxigenin-labeled antisense (E) or sense (F) *rhodopsin* probes. (A, B): kidney; (C, D, E, F): retina. Scale bars, 100 μ m. In situ hybridization were performed following conditions; probe hybridization: 20 h, incubation of anti-digoxigenin antibody: 37°C, 2 h, coloration of NBT-BCIP: 16 h.

sus CRY4, with possible correlation to the acquisition of circadian repressor function.

Transcriptional analysis showed that *XtCRY4* had no inhibitory effect on the E-box-mediated transactivation of *CLOCK/BMAL1*, which was in sharp contrast to the mammalian-type CRYs, *XtCRY1* and *XtCRY2* (Fig. 3). Structurally, this may be due to the lack of a nuclear localization signal (NLS) in *XtCRY4* since nuclear localization is important for CRY repressor activity (Hirayama et al., 2003). Indeed, GFP-*XtCRY4* showed cytoplasmic localization (Fig. 4). CRY4s are likely involved in functions other than circadian transcriptional repressor activity. It is, however, possible that an unidentified factor binds to CRY4 enabling translocation into the nucleus of non-mammalian cells.

The qRT-PCR analysis (Fig. 5) revealed highly expressed *XtCry4* mRNA in the ovary out of the eleven *X. tropicalis* tissues examined in this study. In chickens, *cCry4* mRNA levels are relatively high in the retina, pineal gland, mesencephalon, diencephalon and the kidney (Kubo et al., 2006), which differs from the *Cry4* expression profile where *X. tropicalis*: *XtCry4* levels in the retina or brain were relatively low (Fig. 5). This difference in gene expression profile may be related to the functional divergence in CRY4 proteins.

We also noticed with qRT-PCR analysis that *XtCry4* expression levels showed no or only weak day-night change

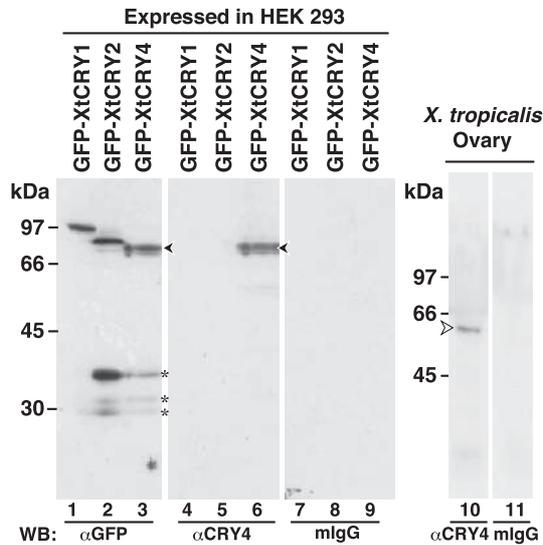


Fig. 10. Specificity of anti-XtCRY4 antibody and immunoblot detection of XtCRY4 in the *X. tropicalis* ovary homogenate. (Left panel) Immunoblot detection of GFP-XtCRY proteins expressed in HEK 293 cells. Each cell lysate was subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting and detection with anti-GFP antibody (α GFP, 900 ng/ml) or anti-XtCRY4CCT antibody (α CRY4, 100 ng/ml) or mouse IgG (mIgG, 900 ng/ml). Expression and loading of all the GFP-fusion proteins were confirmed by detection with the anti-GFP antibody. Positive bands at ~35 kDa with the anti-GFP antibody (asterisks) most likely correspond to degraded GFP. Calculated molecular masses for GFP-XtCRY1, GFP-XtCRY2, and GFP-XtCRY4 are 99.5 kDa, 94.8 kDa, and 94.0 kDa, respectively. (Right panel) Immunoblot analysis of *X. tropicalis* ovary with the anti-XtCRY4CCT antibody. Eight hundred micrograms of the ovary homogenate was loaded in each lane.

in most tissues, including the ovary (Fig. 6). Although the day-night comparison is not sufficient to conclude whether *XtCry4* expression is regulated by circadian clock, it seems likely that neither light signals strongly regulated the *XtCry4* gene expression.

CRYs in the ovary may function as a regulator for the estrus cycle. In situ hybridization analysis indicated that *XtCry4* was expressed in the oocyte throughout most developmental stages (Fig. 8). Therefore, *XtCry4* may be actively transcribed during ovary development or the transcripts may be stably accumulated until a later stage. The former is more plausible, since an intense signal was still observed during later stages (Fig. 8A and C) when the volume of oocytes was much greater. It is interesting to speculate the involvement of CRY4 in oocyte maturation and the present results may have biological relevance to our recent investigation of the lunar- and estrus-cycle-associated expression of *SgCry3* in the brain of a lunar-responder goldlined spinefoot (Fukushiro et al., 2011). Identification of the cells expressing *XtCry4* protein and mRNA would help to answer this question in future.

We also found that all the known *Crys* in *X. tropicalis*, *Cry1*, *Cry2*, and *Cry4*, were highly transcribed in the testis (Fig. 7), and *Cry4* transcripts were accumulated in Leydig cells (Fig. 8). Because Leydig cells are the functional target of pituitary hormone luteinizing hormone and release andro-

gens by its stimulation, CRYs may also play a role in testicular growth or regulation of spermatogenesis.

Recently, CRYs have been suggested to function as a light-dependent magnetoreceptor in *Drosophila* (Gegear et al., 2008; Foley et al., 2011). In migratory birds, CRY in the retina is postulated to operate as a magnetoreceptor by virtue of the spherical structure of the eye (Ritz et al., 2000). In *X. tropicalis*, the mRNA level of XtCRY4 was very low in the retina (Fig. 5), but we did detect mRNA expression in multiple types of neural cells with in situ hybridization (Fig. 9C). Therefore, XtCRY4 may be a candidate for a magnetoreceptor in the frog retina.

We have previously detected relatively higher expression of cCRY4 in the chick pineal gland (Kubo et al., 2006) and retina (Watari et al., 2012). However, in other tissues or animals, there is little information for CRY4 protein expression. To our knowledge, there have been no reports on CRY protein expression in the ovary (including other CRY family proteins such as CRY1 and CRY2 in other animal species). Immunohistochemical analysis using an anti-XtCRY4 antibody was attempted, but the trials resulted in no specific signals in the ovary even when screened under various experimental conditions (data not shown). Instead, immunoblot analysis using the anti-XtCRY4 antibody enabled us to detect novel findings of intrinsic XtCRY4 protein in the ovary (Fig. 10). Ovarian XtCRY4 protein levels were measured as approximately 2×10^6 XtCRY4 molecules per egg (500 μ m in diameter) and under the assumption that XtCRY4 protein was equally expressed at every stage of oocyte development. Thus, ovarian XtCRY4 might function to detect external light signals in the ovary, if it uses the downstream phototransduction cascade like the plant CRY (Liu et al., 2011). The estimated XtCRY4 protein level (~20 ng protein/g tissue) was much lower than cCRY4 expression in the chick retina (~200 ng protein/ chick retina, corresponding to ~5 μ g protein/g tissue; (Watari et al., 2012)) and that of the blue light-sensitive pineal photoreceptor pinopsin (~10 ng/chick pineal gland, corresponding to ~3 μ g/g tissue (Okano et al., 1997)). Either the possible ovarian photoreception would be less sensitive than the pineal or retinal photoreception seen in higher vertebrates, or the putative photoreceptor XtCRY4 might be concentrated in a specific group of oocytes.

In the past few years, an approach for targeted gene disruption using transcription activator-like effector nucleases (TALENs) has been applied to lower vertebrates including the *Xenopus* species (Lei et al., 2012). Potential benefits of *X. tropicalis* as an animal model with the aid of TALEN technology, would enable analysis of the molecular function and role(s) of XtCRY4 proteins and further elucidation of a possible light- and/or estrus-timing-associated mechanism(s) that involves CRY4 and the downstream signal transduction pathways.

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