EMBO reports

scientific report

Diatom PtCPF1 is a new cryptochrome/photolyase family member with DNA repair and transcription regulation activity

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Members of the cryptochrome/photolyase family (CPF) are widely distributed throughout all kingdoms, and encode photosensitive proteins that typically show either photoreceptor or DNA repair activity. Animal and plant cryptochromes have lost DNA repair activity and now perform specialized photoperceptory functions, for example, plant cryptochromes regulate growth and circadian rhythms, whereas mammalian and insect cryptochromes act as transcriptional repressors that control the circadian clock. However, the functional differentiation between photolyases and cryptochromes is now being questioned. Here, we show that the PtCPF1 protein from the marine diatom *Phaeodactylum tricornutum* shows 6-4 photoproduct repair activity and can act as a transcriptional repressor of the circadian clock in a heterologous mammalian cell system. Conversely, it seems to have a wide role in blue-light-regulated gene expression in diatoms. The protein might therefore represent a missing link in the evolution of CPFs, and act as a novel ultraviolet/blue light sensor in marine environments.

Keywords: cryptochrome; 6-4 photolyase; photoreceptor evolution; diatom; DNA repair

EMBO reports (2009) **10**, 655–661. doi:10.1038/embor.2009.59

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Received 22 October 2008; revised 26 February 2009; accepted 26 February 2009; published online 8 May 2009

INTRODUCTION

Light is a source of energy and a crucial environmental signal controlling a range of physiological, adaptive and biochemical processes (Chen *et al*, 2004). Excessive irradiance can also affect the photosynthetic apparatus (Rochaix, 2004), and ultraviolet components of sunlight can damage DNA resulting in mutagenesis and cell death (Sancar, 2003).

In spite of the ecological relevance of marine phototrophs (Falkowski *et al*, 1998), our understanding of light-driven processes in the oceans is still in its infancy. As blue light (350–500 nm) is the prevailing band below the water surface and the only signal travelling long distances within the water column (Kirk, 1983), blue light sensors are expected to be crucial in controlling growth and distribution of prominent eukaryotic microalgae such as diatoms (Smetacek, 1999). Low fluence blue light can indeed be seen to elicit a strong genome-wide response in the marine diatom *Phaeodactylum tricornutum* (M.H., A.F. & C.B., unpublished data; http://www.biologie.ens.fr/diatomics/EST3/), indicating the presence of blue light photoreceptors in this alga.

Cryptochrome blue/ultraviolet-A light photoreceptors regulate growth and circadian rhythms in land plants, whereas in mammals and some insects they act as transcriptional repressors in the circadian clock (Cashmore, 2003; Yuan *et al*, 2007). Cryptochromes have sequence similarity with photolyases, which are flavoproteins that catalyse the repair of ultraviolet-damaged DNA (cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts; Sancar, 2003; Lin & Todo, 2005), thus suggesting a tight evolutionary link between these two groups of biomolecule. It has been proposed that photolyases evolved in Precambrian times as a defence against ultraviolet light and that photoreceptor function differentiated subsequently as a means to control diurnal vertical migrations in the oceans to limit ultraviolet exposure during the day (Gehring & Rosbash, 2003), concomitant with the loss of DNA repair activity. Plant cryptochromes are more similar to CPD photolyases, whereas animal cryptochromes have a closer

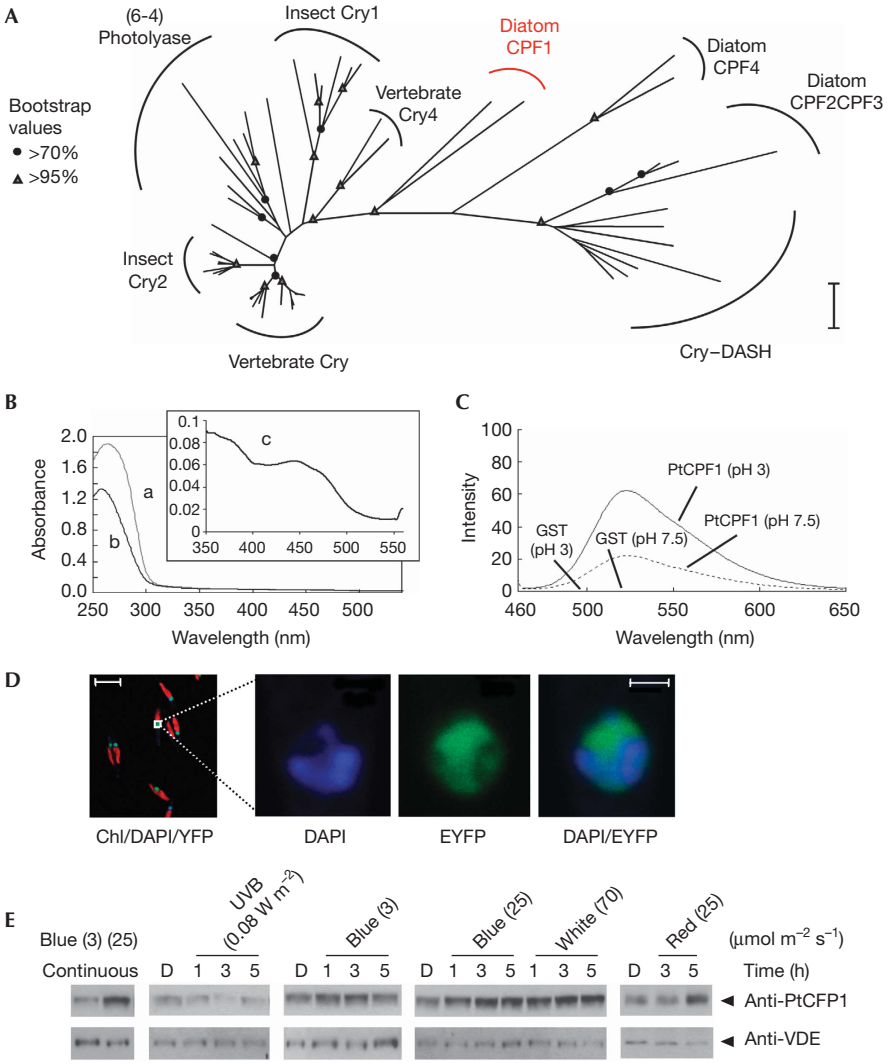

Fig 1 | Phylogenetic and biochemical characterization of *PtCPF1*. (**A**) Phylogenetic analysis of diatom CPF proteins. In total, 52 protein sequences (supplementary information online) were used to perform phylogenetic analysis using the MEGA 4.0 platform with the neighbour-joining method. Bootstrap values of 1,000 replicas were selected together with the JTT model. Scale bar, 0.2 substitutions per site. (**B**) Absorption spectra of purified PtCPF1 (profile a) and of supernatant after the removal of heat-denatured protein (containing nucleic acids, profile b). The inset (c) shows an expanded scale of the absorption spectrum in the 350–560 nm range. (**C**) Fluorescence spectra of heat-denatured PtCPF1 and GST proteins. Excitation was at 450 nm and the emission spectra were taken at pH 3.0 and pH 7.5. (**D**) Fluorescence microscopy of *Phaeodactylum tricornutum* cells expressing amino-terminal EYFP-tagged CPF1. Red corresponds to chlorophyll fluorescence (Chl), blue to DAPI fluorescence and green to EYFP fluorescence. Scan zoom level is 1×100 for left panel (scale bar, 10 μm) and 12×100 for right panels (scale bar, 1 μm). (**E**) PtCPF1 protein level shown by immunoblot using PtCPF1 antiserum in cells grown under various light conditions: continuous blue light (3 and 25 $\mu mol\ m^{-2}\ s^{-1}$), dark (60 h) and then exposed for 5 h to UV-B (0.8 $W\ m^{-2}$), blue (3 or 25 $\mu mol\ m^{-2}\ s^{-1}$), white (70 $\mu mol\ m^{-2}\ s^{-1}$) or red (25 $\mu mol\ m^{-2}\ s^{-1}$) light. The highest level of induction was observed after 3 h in blue 25 $\mu mol\ m^{-2}\ s^{-1}$ (1.95-fold) and in white (2.1-fold) light. PtCPF1 levels were quantified by densitometry as indicated in the supplementary information online. Membranes were hybridized with an antibody VDE (violaxanthin de-epoxidase) as a loading control. CPF, cryptochrome/photolyase family; DAPI, 4,6-diamidino-2-phenylindole; EYFP, enhanced yellow fluorescent protein; GST, glutathione *S*-transferase; Pt, *Phaeodactylum tricornutum;* UV-B, ultraviolet-B.

evolutionary relationship to 6-4 photolyases (Cashmore *et al*, 1999; Lin & Todo, 2005), suggesting that they evolved from photolyases several times independently. The recent characterization of the cryptochrome–DASH family in bacteria (Brudler *et al*, 2003; Kleine *et al*, 2003) has provided the first evidence of the evolution of cryptochromes before the origin of eukaryotes.

The functional differentiation among photolyases and cryptochromes is now being questioned because three photolyases that show regulatory activity have recently been discovered in fungi (Berrocal-Tito *et al*, 2007; Bayram *et al*, 2008; Bluhm & Dunkle, 2008). The generality of this finding is, however, unclear. Here, we characterize a protein from a marine

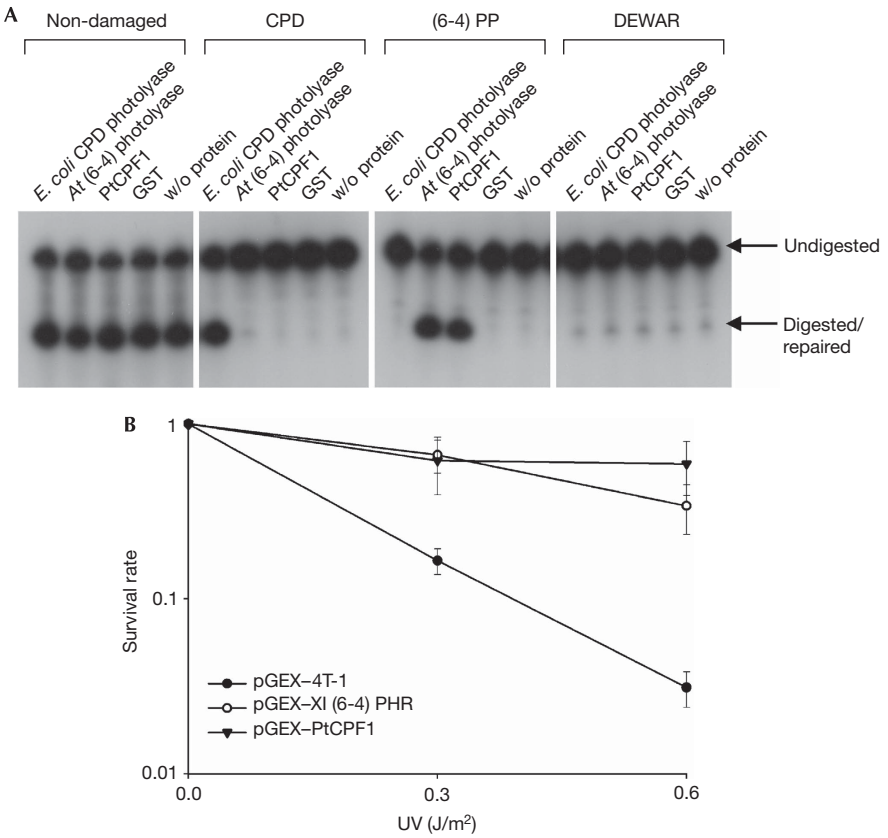

Fig 2 | PtCPF1 DNA repair activity. (**A**) *In vitro* DNA photorepair assay. Purified proteins were incubated with a labelled 49-bp DNA fragment containing a single ultraviolet photoproduct at the *Mse*I restriction site (either a CPD or (6-4) photoproduct). A DEWAR (6-4) photoproduct isomer (which cannot be repaired) and an undamaged DNA probe were used as controls. After photoreactivation, the DNA was digested with *Mse*I and separated on a 10% polyacrylamide gel. Lane l, *Escherichia coli* CPD photolyase; 2, *Arabidopsis thaliana* (6-4) photolyase; 3, PtCPF1; 4, GST; 5, control without protein. (**B**) *In vivo* DNA photolyase assay. Effect of photoreactivation on the survival of ultraviolet-irradiated *E. coli* SY32 cells carrying the vectors pGEX–4T-1 (black circle), pGEX–Xl (6-4) PHR (open circle) and pGEX–PtCPF1 (triangle). Bars indicate standard deviation from three independent experiments. CPD, cyclobutane pyrimidine dimer; CPF, cryptochrome/photolyase family; GST, glutathione *S*-transferase.

diatom that shows both 6-4 photolyase and regulatory activity. Our findings extend the dual functionality of the cryptochrome/photolyase family (CPF) proteins to photosynthetic organisms, which will aid reconstruction of the evolutionary and functional history of this protein family.

RESULTS AND DISCUSSION

Biochemical characterization of diatom PtCPF1

The recent availability of whole genome sequences from two diatom species (Armbrust *et al*, 2004; Montsant *et al*, 2007; Bowler *et al*, 2008) favoured the exploration of photoreceptory mechanisms in marine diatoms. Extended comparative analyses of genes encoding CPF members showed that both diatoms *Thalassiosira pseudonana* and *P. tricornutum* contain a member (CPF1) phylogenetically closer to the animal cryptochrome/6-4 photolyases, but nonetheless forming an independent clade (Fig 1A; supplementary Figs S1 and S2 online). The presence of an animal-type protein can be explained by the evolutionary history of this group of organisms, which are thought to derive from a secondary endosymbiosis between a eukaryotic phototroph and heterotroph and by the fact that many diatom proteins are more similar to animal rather than to plant counterparts (Armbrust *et al*, 2004). Another sequence (CPF4) was also distantly related to other CPFs, suggesting a diatom-specific divergence of the family. Diatom genomes also contain members of the cryptochrome–DASH family (CPF2 and CPF3), for which the DNA repair activity has been clearly shown (Pokorny *et al*, 2008), but evidence to support a photoreceptor function is still very limited (Brudler *et al*, 2003). Surprisingly for photosynthetic algae, neither diatom contains an orthologue of plant cryptochromes.

Putative CPD photolyases, but no clear 6-4 photolyase, could also be identified in each diatom. This was unexpected because 6-4 photolyases are important DNA repair enzymes in eukaryotes (Sancar, 2003). As CPF1 was the only diatom member related to proteins for which photoreceptor and 6-4 photolyase activity has been clearly shown, we characterized the CPF1 protein from *P. tricornutum* (PtCPF1) in more detail.

Recombinant PtCPF1 was purified from *Escherichia coli* as a glutathione *S*-transferase fusion for spectral characterization. The presence of a flavin adenine dinucleotide chromophore was confirmed by the absorption spectra of the heat-denatured protein from 350 to 450 nm (Fig 1B) and the pH-dependent changes in the fluorescence emission spectra (Fig 1C; Sancar, 2003; Daiyasu *et al*, 2004). This evidence showed that PtCPF1 is a

blue-light-absorbing protein. A large absorption peak was also observed at 260 nm, suggesting the presence of nucleic acids, which were further shown to be RNA (supplementary Fig S3 online). The cryptochrome–DASH protein from *Vibrio cholerae* (VcCry1) has also been shown to contain RNA (Worthington *et al*, 2003), although the meaning of this observation is unclear. Purification of PtCPF1 from diatoms will be necessary to establish the functional implications of this finding.

Analysis of transgenic *P. tricornutum* cells overexpressing a yellow fluorescent protein fusion of PtCPF1 (YFP–CPF1) showed the protein to be in the nucleus, and subcellular localization was not differentially regulated by dark, blue or ultraviolet light treatments (Fig 1D and data not shown). PtCPF1 shows only a modest diurnal fluctuation (data not shown), presumably because of higher protein stability. As for the insect cry2 family (Yuan *et al*, 2007), the protein is not light labile. By contrast, PtCPF1 is expressed in the dark and in cells grown under continuous blue light (3 and 25 μmol m^{-2} s^{-1}), and its expression is induced by light (Fig 1E), as already shown for the fungal PHL1 photolyase that also shows regulatory activity (Bluhm & Dunkle, 2008). PtCPF1 levels increase in dark-adapted cells shifted to white and blue light, with a slightly stronger effect at higher intensities (Fig 1E). Red light also moderately induced the expression of PtCPF1, suggesting that the different red and blue light photoreceptors identified in the diatom genomes (Montsant *et al*, 2007) might control its expression. Ultraviolet-B light did not affect PtCPF1 levels.

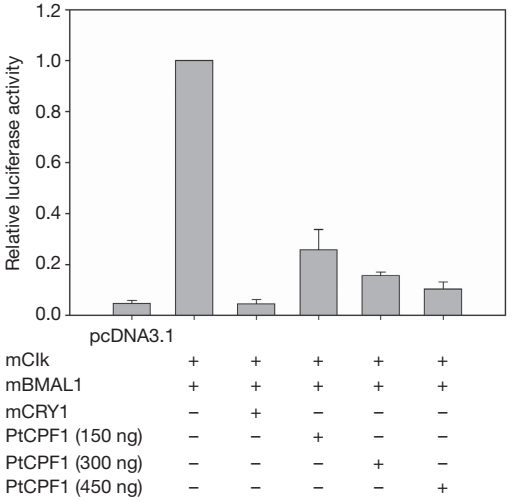

Fig 3 | Transcriptional repressor activity of PtCPF1 in COS7 cells. Inhibition of Clock:BMAL-mediated transcription from the E box bearing reporter plasmid pmAVP-luc by mammalian (m)CRY1 and *Phaeodactylum tricornutum* (pt)CPF1 (supplementary information online). In each experiment, the luciferase activity of the mClk:mBMAL1-containing sample was taken as 100%. Values are mean ± s.e. of three independent experiments. CPF, cryptochrome/photolyase family, mClk, mammalian clock.

PtCPF1 has DNA repair activity

DNA repair activity was first assessed using PtCPF1 recombinant protein. Gel shift assays using double-stranded oligonucleotides containing CPD and 6-4 photoproduct substrates showed that PtCPF1 bound to 6-4 photoproduct-containing DNA but not to CPD-containing oligonucleotides (data not shown). We then used a similar binding assay to show that the protein can also repair DNA, by restoration of an *Mse*I restriction site within the 6-4-photoproduct-damaged DNA (Kobayashi *et al*, 2000; Fig 2A). PtCPF1 therefore shows 6-4 photolyase activity on double-stranded DNA substrates *in vitro* and does not bind to or repair CPD-containing DNA.

Cryptochrome proteins typically do not show photolyase activity (Selby & Sancar, 2006). In the two sequenced diatoms, CPF1 is the only possible member of the 6-4 photolyase family, suggesting that its DNA-repair activity has physiological importance. To examine this, we expressed *PtCPF1* in SY32, a DNA-repair-defective *E. coli* strain that can repair CPDs but not 6-4 photoproducts (Kobayashi *et al*, 2000), and assessed survival rates after exposure to ultraviolet irradiation. The results in Fig 2B show that the expression of *PtCPF1* significantly increased cellular survival in this genetic background to a similar extent as a true 6-4 photolyase from *Xenopus* (Daiyasu *et al*, 2004). We therefore conclude that the 6-4 photolyase activity of PtCPF1 is of functional relevance for cell survival after ultraviolet irradiation.

PtCPF1 transcriptional repressor activity in COS cells

Mammalian cryptochromes are characterized as integral components of the negative transcriptional feedback loop of the circadian clock, acting together with the Period proteins to inhibit the activity of the Clock:BMAL1 heterodimer (Sancar, 2004). This heterodimer normally binds to the E box within the promoters of clock-regulated genes to stimulate their expression. Recently, transcriptional repressor activity has also been shown for the photo-insensitive cry2 protein but is absent in insect 6-4 photolyases (Yuan *et al*, 2007), suggesting that the ability to repress transcription evolved *de novo* in the cryptochrome/animal 6-4 photolyase family from a photolyase ancestor that lacked this function.

Owing to the sequence similarity of the diatom protein to animal cryptochromes (Fig 1A), we tested the ability of PtCPF1 to interact with the mammalian clock machinery using a transcription assay developed in COS7 cells (Kume *et al*, 1999). We found that PtCPF1 can effectively repress Clock:BMAL1-regulated expression of a reporter gene (Fig 3), which has never been observed for a protein showing 6-4 DNA photolyase activity (Kobayashi *et al*, 2000; Yuan *et al*, 2007). Reiteration of transcriptional repressor activity by PtCPF1 in this heterologous system is remarkable considering the wide divergence of animals and diatoms, and might indicate either an ancient origin of this activity or convergent evolution. A general effect on the transcriptional machinery was excluded by performing the same assay with a construct containing an SV40 promoter (supplementary Fig S4 online).

Transcriptional regulation of diatom genes by PtCPF1

We examined the possible role of PtCPF1 in transcriptional regulation in *P. tricornutum* by analysing blue-light-regulated gene expression in wild type and two *PtCPF1*-overexpressing lines (OE1 and OE2), which express approximately 25 and 35 times the wild-type levels of PtCPF1 protein (Fig 4; supplementary Fig S5 online). Specifically, dark-adapted cells were exposed to a 5-min pulse of low-intensity blue-light (3.3 μmol m^{-2} s^{-1}) to favour photoreceptor-mediated events rather than retrograde signalling

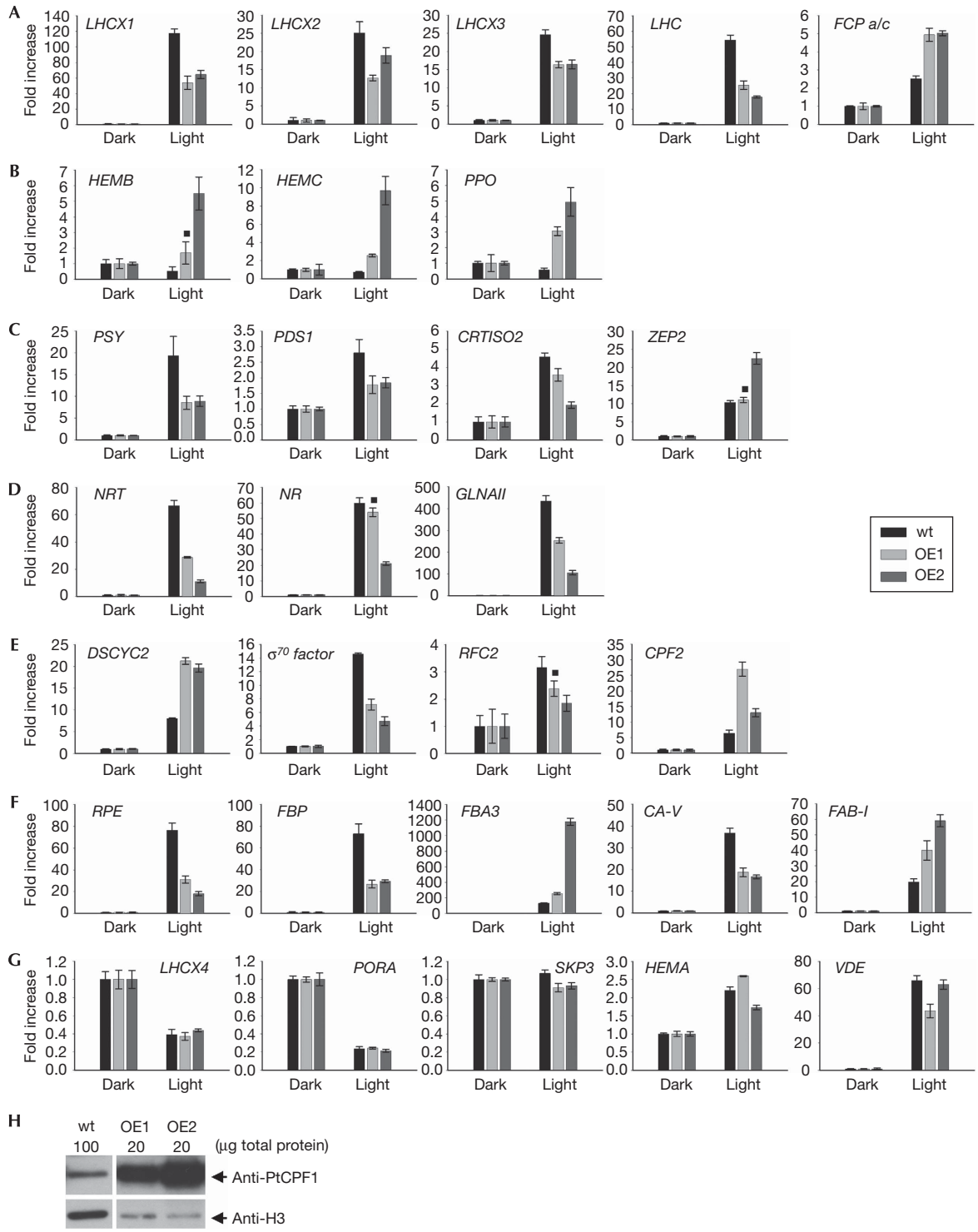

Fig 4 | Regulation of diatom gene expression by PtCPF1. Relative expression levels in wild type (wt; black bar) and two independent *PtCPF1*-overexpressing lines (OE1 and OE2 represented by light grey and dark grey bars, respectively) of genes putatively involved in different cellular processes. (**A**) Photosynthetic light harvesting, (**B**) tetrapyrrole biosynthesis, (**C**) carotenoid biosynthesis, (**D**) nitrogen metabolism, (**E**) genetic information, (**F**) miscellaneous and (**G**) genes unaffected by PtCPF1. Dark-adapted cultures (60 h) were exposed for 5 min to 3.3 µmol m^{-2} s^{-1} blue light, and relative transcript levels were determined after 15 min by qRT–PCR using *RPS* as a reference gene. Values were normalized to gene expression levels in the dark. Squares indicate experiments that did not pass the statistical *t*-test. Error bars represent standard deviations. (**H**) Immunoblot analysis of PtCPF1 protein levels in wild type, and OE1 and OE2 lines (supplementary Fig S5 online). H3 protein level was detected as a loading control. qRT–PCR, quantitative reverse transcriptase–PCR; VDE, violaxanthin de-epoxidase.

from photosynthetic activity. We then analysed genes that are known to be regulated by blue-light photoreceptors in plants (Casal & Yanovsky, 2005; Jiao *et al*, 2007 and references therein) and algae (Lohr *et al*, 2005; Coesel *et al*, 2008), or that were expressed in a *P. tricornutum* expressed sequence tag library derived from blue-light-exposed cells (http://www.biologie.ens.fr/diatomics/EST3/), and examined their expression by quantitative reverse transcriptase–PCR (qRT–PCR). Genes were also selected on the basis of a preliminary analysis of global gene expression in wild-type and OE2 cells using a first-generation *P. tricornutum* whole transcriptome microarray (supplementary Table S1 online and data not shown).

These analyses show that *PtCPF1* overexpression differentially affected genes involved in distinct cellular pathways and in most cases these effects correlated with the different overexpression levels in OE1 and OE2 cell lines (Fig 4; supplementary Table S1 online).

PtCPF1 overexpression antagonistically affected the blue-light-induced expression of genes encoding putative light-harvesting proteins (*LHCX*), such as three L1818-type genes. Genes encoding the carotenoid biosynthetic enzymes, *PDS* and *PSY*, were similarly downregulated, whereas several genes encoding tetrapyrrole biosynthetic enzymes were upregulated. These effects infer that PtCPF1 might perform a regulatory role in controlling photosynthetic light harvesting and photoprotection. Consequently, in spite of their separate origins, both plant cryptochrome photoreceptors and the animal-like PtCPF1 might be involved in the regulation of processes typical of photosynthetic organisms, suggesting their independent convergent evolution in these eukaryotes.

Also, the blue-light-induced expression of genes involved in nitrogen metabolism, such as *NR*, *NRT* and *GLNAII* was significantly dampened in OE1 and OE2, whereas expression of the putative cryptochrome–DASH photoreceptor *PtCPF2* was enhanced (Fig 4), supporting a general role for PtCPF1 in the regulation of blue-light-sensing mechanisms. Genes involved in cell-cycle progression and DNA repair were also found to be affected by *PtCPF1* overexpression (Fig 4). Both photolyase and photoreceptor activity of PtCPF1 could be relevant during cell division, for example, in the maintenance of timing of cell division at night, as is typical of marine algae (Vaulot *et al*, 1986).

A general effect of PtCPF1 on transcription was excluded because many genes were unaffected in OE1 and OE2 (Fig 4; supplementary Table S1 online; microarray data, M.M., C.B. & A.F., unpublished data). In addition, in contrast to blue light, PtCPF1 did not affect red light responses (data not shown; supplementary Fig S5 online). The observed effects are therefore consistent with PtCPF1 acting as a photoreceptor for blue light, further reinforced by the fact that transcript levels of selected genes were specifically controlled by blue light through PtCPF1 in a fluence-rate-dependent manner, as already shown for plant cryptochromes (supplementary Fig S5 online; Chen *et al*, 2004).

Conclusions

The biochemical and functional characterization of *PtCPF1* has shown it to be a new blue light sensor with 6-4 photolyase and blue-light-dependent transcription regulation activity. As no CPF proteins with dual function have previously been discovered in photosynthetic organisms, the PtCPF1 might fill a missing link in the evolution of this protein family. PtCPF1 is most likely a result of independent evolution and function diversification in marine eukaryotes, and opens the way for molecular evolution studies, for example, to understand how catalytic activity evolved from a photolyase to a photoreceptor. Further studies are necessary to identify crucial residues for both functions and to determine specific light conditions under which PtCPF1 shows DNA repair and/or photosensory roles in diatoms. The spectral characterization of an orthologous protein from the marine green alga *Ostreococcus tauri* has recently been reported (Usman *et al*, 2009). In contrast to animal cryptochromes, the regulatory function of PtCPF1 not only seems to be based on transcriptional repressor activity within the circadian clock but also acts as a more general regulator of blue light perception and signalling. CPF1 might link diurnal or circadian rhythms to cell-cycle progression and to DNA-damage checkpoints, which is an attractive hypothesis to explain the evolutionary origin of circadian clocks. Identification of diatom clock components and the generation of *CPF1* knockdown mutants will be required to formally assess this possibility.

The presence of a single protein with dual function might also have been an important factor underlying the emergence of diatoms to their ecological prominence in contemporary oceans. Recent microbial community gene expression analyses have shown that photolyase genes are highly expressed in oceanic surface waters (Frias-Lopez *et al*, 2008), so continued analyses in other phytoplanktonic organisms will help to assess the generality of dual function of this protein family in these environments.

METHODS

Cell culturing. *P. tricornutum* (CCMP2560) was obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton and grown in f/2 medium (Guillard, 1975) at 18 °C in a 12 h photoperiod (white light intensity 100 $\mu mol\ m^{-2}\ s^{-1}$). PtCPF1 expression analyses were also performed on cells grown under various light conditions, as specified in the text.

PtCPF1 characterization. PtCPF1 was identified in the *P. tricornutum* genome (Protein ID 27429, http://genome.jgi-psf.org/Phatr2/Phatr2.home.html), amplified by qRT–PCR and inserted in different bacterial and diatom expression vectors for biochemical and functional analyses, as indicated in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank A. Manfredonia and Stazione Zoologica Molecular Biology Service for technical assistance, M. Oborník (University of South Bohemia, Branisovská) for phylogenetic analysis advice, F. Formiggini (CEINGE, biotecnologie avanzate, Naples) for microscopy assistance, K. Tessmar-Raible (Vienna Biocenter) and M. Ribera D'Alcalà (Stazione Zoologica of Naples) for critical suggestions. This study was supported by the Human Frontier Science Programme-Career Development Award to A.F. (0014/2006), Agence Nationale de la Recherche to C.B., EU-FP6 Marine Genomics Europe (GOCE-CT-2004-505403) and EU-FP6 Diatomics (LSHG-CT-2004-512035). C.B. and A.F. contributed equally to the coordination of this work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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