

Screening by coral green fluorescent protein (GFP)-like chromoproteins supports a role in photoprotection of zooxanthellae

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Abstract Green fluorescent protein (GFP)-like pigments are responsible for the vivid colouration of many reef-building corals and have been proposed to act as photoprotectants. Their role remains controversial because the functional mechanism has not been elucidated. We provide direct evidence to support a photoprotective role of the non-fluorescent chromoproteins (CPs) that form a biochemically and photophysically distinct group of GFP-like proteins. Based on observations of *Acropora nobilis* from the Great Barrier Reef, we explored the photoprotective role of CPs by analysing five coral species under controlled conditions. *In vitro* and *in hospite* analyses of chlorophyll excitation demonstrate that screening by CPs leads to a reduction in chlorophyll excitation corresponding to the spectral properties of the specific CPs present in the coral tissues. Between 562 and 586 nm, the CPs maximal

absorption range, there was an up to 50 % reduction of chlorophyll excitation. The screening was consistent for established and regenerating tissue and amongst symbiont clades A, C and D. Moreover, among two differently pigmented morphs of *Acropora valida* grown under identical light conditions and hosting subclade type C3 symbionts, high CP expression correlated with reduced photodamage under acute light stress.

Keywords Green fluorescent protein (GFP)-like proteins · Chromoproteins · Function · Photoprotection · Growth · Coral-zooxanthellae symbiosis

Introduction

Corals inhabit a range of light environments spanning two orders of magnitude of irradiance from shallow waters to deeper mesophotic reefs (Lesser et al. 2010). Consequently, corals and their symbionts need mechanisms for acclimation and adaptation to different irradiances, chromaticity and temporal variability in the light field. Even at the scale of a single shallow-water colony, there are large variations in light exposure of the coral's tissues controlled by factors such as seasonal and diel changes in surface irradiance, changes in the absorbing and scattering materials in the water column, orientation of the tissue surface relative to the incident irradiance and wave-focusing effects (Falkowski et al. 1990; Veal et al. 2010; Kaniewska et al. 2011). The shallow-water environments where reef-building corals thrive are typified by irradiances often greater than photon fluxes of $\sim 300\text{--}750 \mu\text{mol m}^{-2} \text{s}^{-1}$ that saturate photosynthesis of the algal symbionts *in hospite* (Gorbunov et al. 2001; Levy et al. 2004; Hennige et al. 2008). As such, some shallow-water corals live in

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habitats where light is in excess. In addition, other factors, such as temperature stress, may reduce the photosynthetic capacity of the zooxanthellae thus increasing the excess excitation energy (Iglesias-Prieto et al. 1992; Warner et al. 1996; Smith et al. 2005). Exceeding the capacity of the photosynthetic machinery of the algal symbionts can result in photoinhibition and production of reactive oxygen species (ROS) (Smith et al. 2005) that cause damage to cellular components (Lesser 2006). Consequently, both the coral and the symbionts possess mechanisms to reduce light stress. In fact, light stress in combination with elevated water temperatures is considered a fundamental cause of coral bleaching due to increased ROS production and/or inhibition of photosystem II (PSII) repair mechanisms (Tchernov et al. 2004; Smith et al. 2005; Lesser 2006; Takahashi et al. 2009). Furthermore, light is a key driver of nutrient-stress-mediated coral bleaching (Wiedenmann et al. 2012).

Corals' endosymbiotic algae optimise light absorption and usage in response to incident irradiance. The mechanisms employed include long-term acclimation strategies such as changes to the size and/or number of photosynthetic units (PSUs) (Falkowski and Dubinsky 1981; Hennige et al. 2009) as well as acclimatory mechanisms designed to address the supraoptimal irradiances brought about by short-term changes such as diurnal variability. The short-term responses include downregulation of PSII reaction centres (Gorbunov et al. 2001), xanthophyll conversion (Brown et al. 1999), cyclic electron transport and dissociation of antenna complexes from PSII (McCabe Reynolds et al. 2008). The degree to which different acclimatory mechanisms are employed, and their relative contribution to photoprotection, appears to be, at least in part, associated with the genetic identity of the symbiont clade, and hence, there is evidence that some symbiont clades/subclades are better suited to certain environments than others (Iglesias-Prieto et al. 2004; McCabe Reynolds et al. 2008). Nevertheless, in spite of extensive protection afforded by non-photochemical quenching, photodamage to PSII can occur in shallow-water corals on cloud-free days (Gorbunov et al. 2001) and under thermal stress (Warner et al. 1999).

While some host-based responses to light stress are the same as those employed by the symbiont, such as accumulation of MAAs and antioxidant enzymes (Lesser 2006), others remain unique to the host. A photoprotective function has been proposed for the host-based family of green fluorescent protein (GFP)-like pigments that are frequently expressed by anthozoans (Salih et al. 1998, 2000; Wiedenmann et al. 1999; Dove et al. 2001). The host pigments of corals and other anthozoans can be divided into two major groups: the fluorescent proteins (FPs), responsible for cyan to red hues and the non-fluorescent

chromoproteins (CPs; synonym: pocilloporins), that produce the intense pink, purple and blue pigmentation (Dove et al. 2001; Salih et al. 2006; Alieva et al. 2008; D'Angelo et al. 2008). The CPs and FPs are thought to protect the symbionts from high irradiances by absorbing photons or distributing them away from the algal pigments by re-emission or reflectance (Salih et al. 1998, 2000, 2006; Wiedenmann et al. 1999; Dove et al. 2001). It is proposed that these pigments act through screening (Salih et al. 2000), a process whereby the light received by the photosynthetic pigments has passed through a layer or compartment containing the photoprotective pigments (Merzlyak et al. 2008). As such, screening is distinct from other photoprotective systems where the photoprotectants and the photosynthetic pigments directly compete for light absorption (Merzlyak et al. 2008).

The high abundance of fluorescent colour morphs in shallow water, the resistance of their zooxanthellae against photoinhibition, the stability and slow turnover of the GFP-like pigments and their light-regulated expression support a photoprotective function (Wiedenmann et al. 1999; Salih et al. 2000; Leutenegger et al. 2007a; D'Angelo et al. 2008). Nevertheless, the functional mechanism of photoprotection by GFP-like proteins is controversial as no reduction due to GFP absorption was evident in the chlorophyll excitation spectrum of zooxanthellae in Caribbean corals (Mazel et al. 2003). Also, high-level expression of GFP-like proteins in zooxanthellae-free coral growth zones and in several azooxanthellate anthozoans from low-light habitats, including the deep sea, sheds doubts on whether all of these pigments fulfil a photoprotective function (Wiedenmann et al. 2004a; D'Angelo et al. 2008; Vogt et al. 2008; D'Angelo et al. 2012). In addition to questions about the photoprotective role of FPs, the direct effects of CPs on symbiont photosynthesis remain unclear because the CP absorption peaks are outside the photosynthetic pigments' major absorption bands (D'Angelo et al. 2008; Dove et al. 2008). These observations together with the results of molecular phylogenetic analyses of the protein sequences suggest that distinct groups of GFP-like proteins could fulfil various functions among different taxonomic groups (Alieva et al. 2008; D'Angelo et al. 2008, 2012).

We observed blue morphs of *Acropora nobilis* with notably higher zooxanthellae and photosynthetic pigment concentrations than their brown counterparts growing adjacently in a shallow-water reef setting at Heron Island (Electronic Supplemental Material (ESM) Methods, Fig. S1). The increased density and pigment content of the zooxanthellae could be interpreted as a shade acclimation due to screening by the blue chromoprotein, which is the dominant host pigment in these individuals. This observation was the motivation for our study of the role of CPs in coral photobiology. Considering CPs as a key group of

GFP-like proteins that are biochemically and photophysically distinct from their fluorescent counterparts (Alieva et al. 2008; Nienhaus and Wiedenmann 2009), we set out to address three questions: Do CPs screen certain wavelengths of visible light that would otherwise be absorbed by the zooxanthellae photosynthetic pigments? Are the symbionts screened by CPs less susceptible to high irradiances in the relevant spectral range? Is screening relevant to growth zones where symbiont densities are low?

Methods

Coral collection and culture

Collection

Field samples (branch fragments ~ 10 cm length) of brown and blue morphs of *Acropora nobilis*, growing adjacently, were collected at ~ 1.5 m depth at low tide on the outer reef slope of Heron Island, Great Barrier Reef (Marine Parks Permit No. G26894.1) selecting the most light-exposed branches for analysis.

Culture

Five coral species from the Coral Reef Laboratory mesocosm at the National Oceanography Centre Southampton (D'Angelo and Wiedenmann 2012) were selected based on their CP expression: *Acropora valida*, *Acropora polystoma*, *Acropora millepora*, *Seriatopora hystrix* and *Montipora foliosa*. CP-pigmented morphs of *A. valida*, *A. millepora* and *S. hystrix* were analysed for comparison of the optical properties of light-exposed and shaded tissue. *A. valida* colonies were also used to compare photophysiological responses to light stress between the brown and purple morphs. *M. foliosa* and *A. polystoma* were incorporated into the study to explore the effects of localised CP expression in growth zones.

Seriatopora hystrix (D'Angelo et al. 2008) and *A. polystoma* (D'Angelo et al. 2012; Wiedenmann et al. 2012) of unconfirmed geographical origin were purchased from the ornamental trade. The other species were collected from Fijian reefs and acquired through the Tropical Marine Centre (London, UK) (D'Angelo et al. 2012). Corals were acclimated to tank conditions for at least 18 months prior to the experiments. Briefly, corals were grown under a 12-h light:dark cycle provided by 250-W metal halide lamps (ESM Fig. S2; Aqualine 10000 (13,000 K), Aqua Medic, Germany), with the temperature kept at 24 °C (±0.5 °C) during the experiments.

Fragments of *A. valida*, *A. millepora* and *S. hystrix* were grown perpendicular to the incident irradiance for

>1 month to enable acclimation to the light field and induce a localised expression of CPs in the light-exposed parts of the colonies. Measurements with a Li-Cor LI-250A indicated that the upper sides of the coral branches were exposed to photon fluxes of ~200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The underside of the branches received ~10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of reflected photons.

Characterisation of zooxanthellae

Zooxanthellae densities and pigment content were determined using standard protocols (Parsons and Strickland 1963; Marsh 1970; Jeffrey and Humphrey 1975; Leutenegger et al. 2007b). The dominant zooxanthellae clade in the purple and brown morphs of *A. valida* was determined by restriction fragment length polymorphism (RFLP) analyses of the amplified small-subunit ribosomal-DNA (SSU rDNA) (Hartle-Mougiou et al. 2012). Analyses of the PCR amplified internal-transcribed spacer region 2 (ITS2) (LaJeunesse 2002) identified the dominant symbiont subclade (Clade C3; Genbank accession numbers from KC405646 to KC405651) of *A. valida* colour morphs used in the light stress assay.

Reflectance

Coral reflectance spectra, of *S. hystrix*, *M. foliosa* and *A. polystoma*, were recorded (Mazel et al. 2003) using a fibre-optic reflectance probe connected to an Ocean Optics USB4000 spectrometer and a tungsten halogen HL-2000-HP light source (Ocean Optics, USA) with a Spectralon 99 % diffuse reflectance standard as a reference (Lab-sphere, USA). Five independent reflectance (R) measurements were taken from different locations on each colony and used to estimate absorbance (D_e) using the formula $D_e = \log(1/R)$ (Enriquez et al. 2005).

Fluorescence spectroscopy

Chlorophyll excitation spectra were measured as fluorescence excitation of PSII (680 nm) emission using a Varian Cary Eclipse fluorescence spectrophotometer with a fibre-optic attachment (Leutenegger et al. 2007a; D'Angelo et al. 2008). Chlorophyll excitation spectra were recorded and analysed as per ESM methods.

CP purification and quantification

CPs were enriched by isopropanol precipitation (Wiedenmann et al. 2000) and further purified by size-exclusion chromatography, where required (Wiedenmann et al. 2002). The CP spectra were measured on a Cary absorption spectrophotometer (Varian, USA). Three CPs were cloned

from *A. millepora* (Genbank accession: KC349891, KC411499, KC411500) (D'Angelo et al. 2008). Together with asCP562 from *Anemonia sulcata* (Genbank Accession: AF322222) (Wiedenmann et al. 2000), the pigments were recombinantly expressed and purified (Wiedenmann et al. 2002). CP concentrations were quantified as described in ESM methods.

In vitro CP screening

An in vitro experiment was performed to assess the potential for CPs to screen zooxanthellae in the absence of confounding variables.

Symbionts were isolated from *A. sulcata* (clade A) (Visram et al. 2006) and two *A. valida* colonies (clade C and D), washed and resuspended in sterile filtered seawater. Freshly isolated zooxanthellae were loaded into an 8-well culture chamber and allowed to settle for 15 min. Two-millimetre-deep chambers containing CPs and a phosphate-buffered saline (PBS) control were placed between the settled symbionts and the fluorescence spectrometer's fibre-optic probe and chlorophyll excitation spectra were recorded. The CP/PBS filter was used on the same population of the respective clade to ensure comparisons of identical populations with the same physiology and photobiology. The zooxanthellae concentrations (1.0×10^6 cells per cm^{-2} of the bottom of the wells) used in this experiment are comparable to the symbiont density of the blue morphs of *Acropora nobilis* at ~ 1.5 m at Heron Island (ESM Fig. S1). The CPs selected for analysis were asulCP562 (Wiedenmann et al. 2000), amilCP580 and amilCP604 to enable demonstration of the impact of CPs over their range of absorption peaks. The concentrations of each CP filter were calculated to provide the same peak absorbance as the areal tissue concentrations of CP found in a purple morph of *A. valida* (0.09 mg cm^{-2}).

Light stress experiment

Brown and purple morphs of *A. valida*, both hosting subclade C3 symbionts and grown under a photonflux of $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ on the light-exposed sides, were subjected to an additional $1,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ of orange light (peak spectral irradiance = 595 nm; ESM Fig. S2) provided by an LED array (P4 Amber Star, Seoul Semiconductor, Korea) for 2 days with a 12:12 h light:dark cycle. Chlorophyll fluorescence parameters were recorded using a Diving PAM including exposure to dim light ($<5 \mu\text{mol m}^{-2} \text{ s}^{-1}$) prior to and during measurements taken after 12 h dark acclimation to minimise the impacts of chlororespiration (Warner et al. 2010). The PSII maximum quantum efficiency (Fv/Fm) was recorded prior to the experiment and at the end of the second dark cycle. The

effective quantum yield ($\Delta F'/Fm'$) was measured at the end of the second light cycle. Due to the slow relaxation time of chronic photoinhibition (Gorbunov et al. 2001), any reduction in Fv/Fm from the start of the experiment to the end of each dark cycle should indicate PSII damage.

CP expression in growth zones

A. polystoma and *M. foliosa* were used to assess the effects of CPs in growth zones as CP expression is localised to these regions in these species. Approximately 1 cm^2 of tissue was removed from replicate colonies of *A. polystoma* to assess CP expression in regenerating tissue. Half of the colonies were moved to low-light conditions ($\sim 60 \mu\text{mol m}^{-2} \text{ s}^{-1}$), whereas others were left under higher light intensities ($\sim 200 \mu\text{mol m}^{-2} \text{ s}^{-1}$). After 10 days, spectroscopic characterisations of the regenerating areas were made and tissue samples were analysed for protein and zooxanthellae content.

Replicate colonies of two colour morphs of *M. foliosa* were grown under $\sim 150 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The purple morph expresses the CP, mfolCP577, exclusively in the growth margins of the colony, whereas CP expression is essentially absent from the brown morph (D'Angelo et al. 2012). Spectroscopic measurements were taken on both the growth margins and centres of the colonies.

Results

CP spectral properties

The CPs isolated from the studied coral species had absorption peaks ranging from 562 to 586 nm (ESM Fig. S3). The pink appearance of *S. hystrix* is due to the accumulation of shysCP562 with an absorption maximum at 562 nm (D'Angelo et al. 2008), the shortest wavelength of this set of CP pigments. The *M. foliosa* and *A. valida* colonies contain CPs with peak absorptions at 577 and 580 nm, respectively, (herein referred to as mfolCP577 (D'Angelo et al. 2012) and avalCP580). Consequently, the pigmented regions of the corals appear purple-violet. We cloned three CPs from *A. millepora* (amilCP580, amilCP586 and amilCP604) with absorption maxima at 580, 586 and 604 nm. The absorption spectrum of partially purified tissue extracts from a blue *A. millepora* morph peaked at 586 nm suggesting that the blue colour of the coral results from a dominant expression of amilCP586. The molar extinction coefficients were approximated for two proteins, avalCP580 ($183,000 \text{ M}^{-1} \text{ cm}^{-1}$) and apolCP584 ($103,000 \text{ M}^{-1} \text{ cm}^{-1}$). Absorption spectra and molar extinction coefficients are typical of anthozoan CPs (Alieva et al. 2008).

In vitro CP screening

The three *Symbiodinium* clades showed similar chlorophyll excitation spectra with major peaks at around 460 and 525 nm (Fig. 1). While the excitation spectra for clades C and D were almost identical, the contribution of the 525 nm peak in the clade A spectra was reduced, with a maximum difference between them at ~ 520 nm. The latter was likely due to differences in the zooxanthellae pigment composition as this peak has been attributed to peridinin (McCabe Reynolds et al. 2008). When a CP solution was placed between the zooxanthellae and the fibre-optic probe, mimicking the localisation and concentration of CPs in pigmented coral tissue, there was a clear decrease in chlorophyll excitation by up to 18 % compared to the 400–620 nm spectrum. The wavelength-specific reduction was up to 55 % at 580 nm for amilCP580. This decrease in chlorophyll excitation was consistent with the CP absorption characteristics, with excellent agreement between the difference spectra and the CP absorption spectra for all three clades.

Impact of CPs on the optical properties of *S. hystrix*

To confirm the applicability of our in vitro experiment, we performed measurements on a pink colony of *S. hystrix* that expressed a CP absorbing at 562 nm (Fig. 2). In agreement with the in vitro study, the presence of a CP in light-

exposed tissue resulted in decreased chlorophyll excitation corresponding to the CP absorption properties.

The reflectance spectra of the upper and lower sides of the *S. hystrix* colony fall into the two categories described by Hochberg et al. (2004) for corals in the field. The shaded undersides yielded reflectance spectra showing maxima at around 575, 600 and 650 nm typical of “brown” corals (Hochberg et al. 2004). In contrast, the reflectance spectrum of light-exposed pigmented tissue showed features characteristic of “blue” corals: the 575 nm maximum was absent, resulting in a plateau-like shape between 600 and 650 nm (Hochberg et al. 2004). Converting these data into estimated absorbance highlights a maximum at ~ 555 nm for the light exposed side. Comparisons of the difference between the absorbance of the upper and lower tissues with the absorption spectrum of shysCP562 reveal a strong similarity between the feature at ~ 566 nm in the difference spectrum and the CP absorbing at 562 nm.

Relationship between excitation difference and CP absorption

To further investigate CP-mediated screening of symbionts *in hospite*, we compared the excitation difference spectra of light-exposed versus shaded sides, against the absorption properties of the respective CP in *S. hystrix*, *A. valida* and *A. millepora* (Fig. 3). Not only did the shapes of the difference spectra match the CP absorption spectra but the

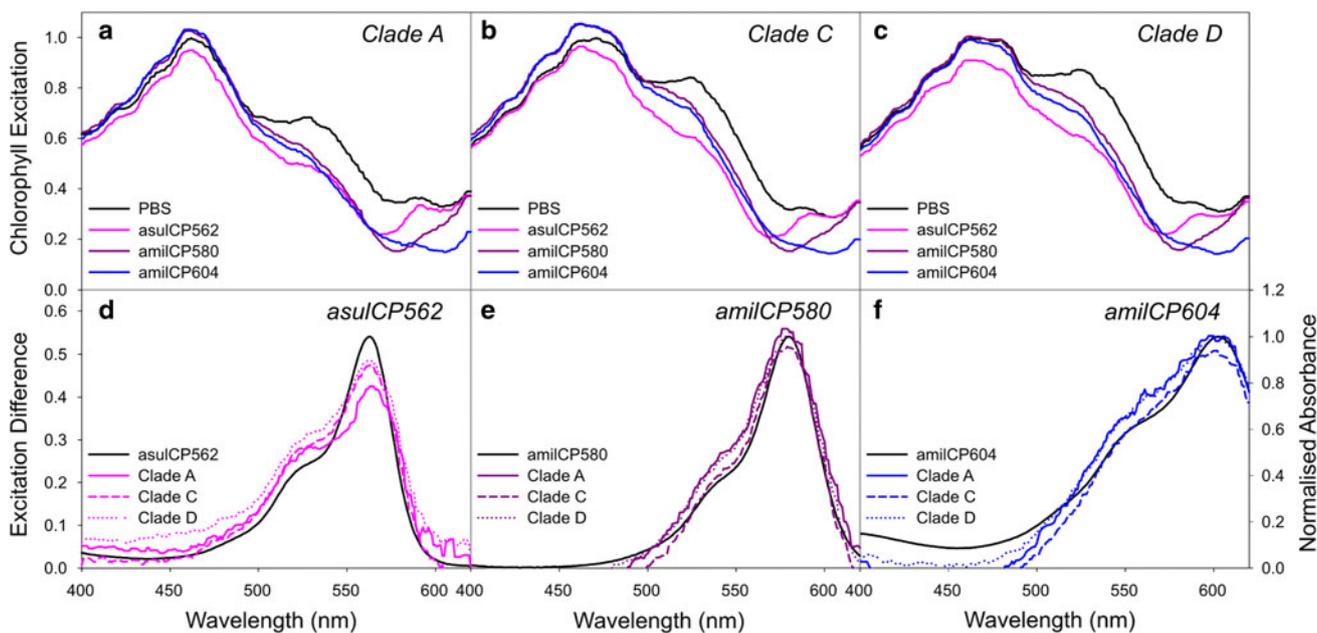


Fig. 1 In vitro assessment of chromoprotein screening. Chlorophyll excitation spectra of freshly isolated zooxanthellae of clades A (a), C (b) and D (c) through solutions of PBS, asulCP562, amilCP580 and amilCP604. Fluorescence spectra are normalised to the PBS excitation spectrum peak. Excitation difference spectra through the

asulCP562 (d), amilCP580 (e) and amilCP604 (f) solutions from clade A, C and D zooxanthellae. The absorption spectra of the respective CPs are shown for reference and are normalised to a peak value of 1

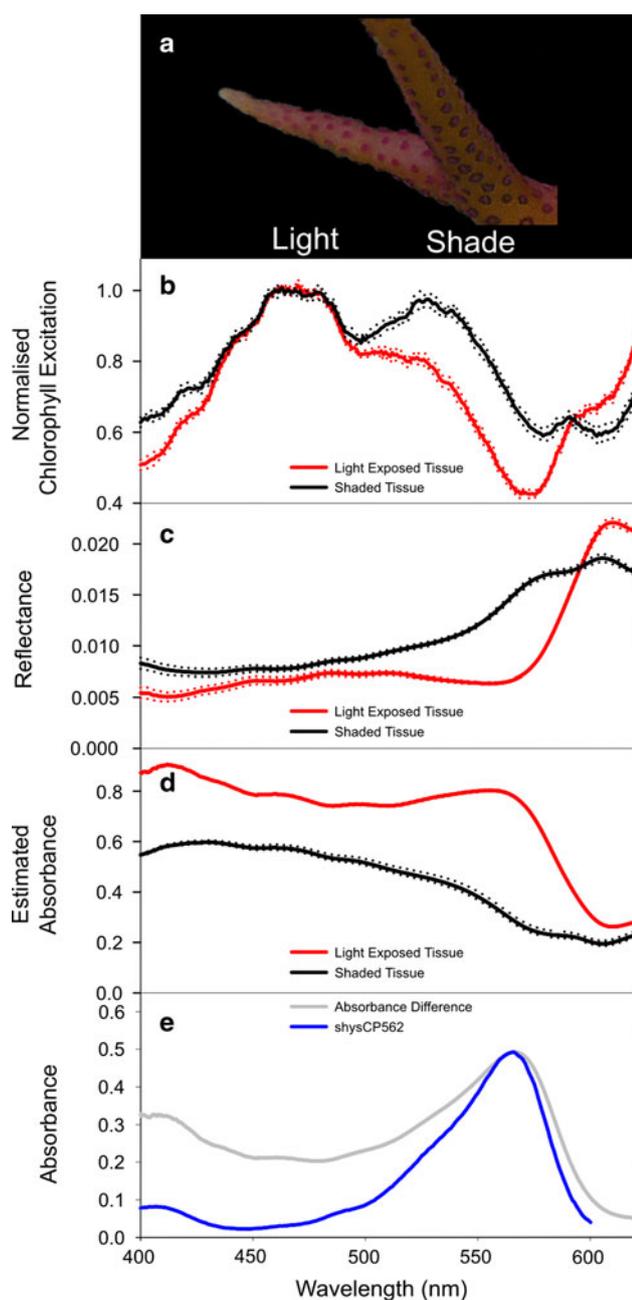


Fig. 2 Impact of shysCP562 on the optical properties of *S. hystrix*. **a** Photograph showing the accumulation of shysCP562 in the light-exposed tissues of *S. hystrix*. **b–d** Normalised chlorophyll excitation (**b**), reflectance (**c**) and estimated absorbance (**d**) spectra of the light-exposed and shaded surfaces. *Solid lines* are the mean of five measurements, with the \pm SD indicated by *dotted lines*. **e** The difference between absorbance of light-exposed and shaded tissues. The absorption spectrum of shysCP562 is shown for reference

peak locations showed good agreement across the 24 nm range of the CPs' absorption maxima (562–586 nm). By compiling the data from five species and different types of comparisons (light vs shaded tissues, growth vs non-growth zones and CP-pigmented vs non-pigmented morphs), we

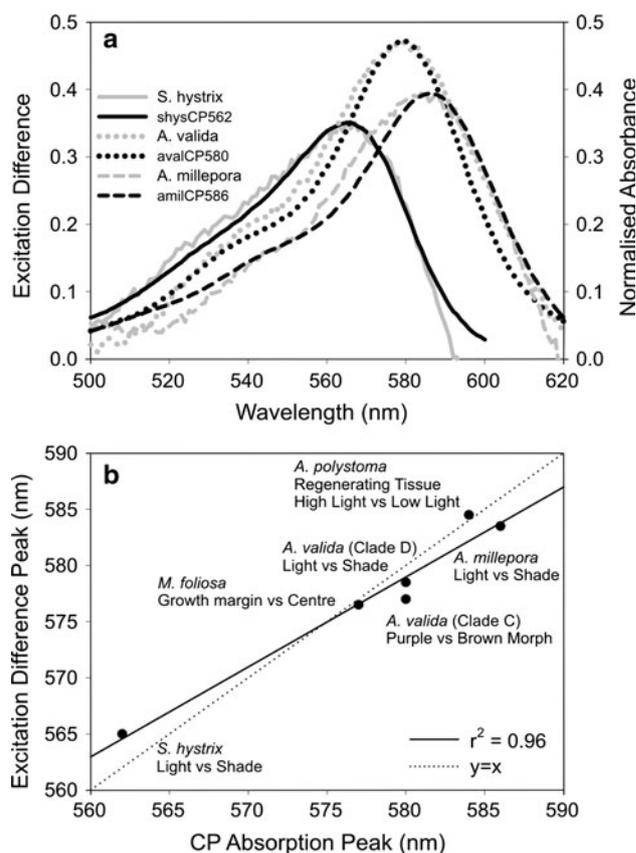


Fig. 3 The relationship between the excitation difference spectra and the respective CP absorption. **a** Comparison between the excitation difference spectra of *S. hystrix*, *A. valida* and *A. millepora*, and the respective CPs (shysCP562, avalCP580, amilCP586) absorption spectra. **b** Linear relationship between the CP absorption peak and the peak of the excitation difference spectrum. *Each point* represents the comparison between pigmented and non-pigmented tissue from five coral species (*S. hystrix*, *A. valida*, *A. millepora*, *A. polystoma* and *M. foliosa*). *A. valida* is represented twice from comparisons between *light* and *shaded tissue* of a *purple* morph hosting clade D symbionts and between the *brown* and *purple* morphs hosting clade C symbionts

identified a strong linear relationship ($r^2 = 0.96$) between the peak of excitation difference spectra and the CP absorption maxima.

Response of colour morphs to high irradiances

Using the purple and brown morphs of *A. valida*, we compared the response of CP-pigmented and non-CP-pigmented tissues to high irradiances to explore the impacts of CP screening on the light stress response of zooxanthellae (Fig. 4). Both colour morphs host subclade C3 zooxanthellae as the dominant symbiont strain. Prior to experimentation, the corals were grown side-by-side at an orthogonal angle to the incident light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) to ensure that they experienced comparable light histories. As CPs

are upregulated in response to light, they are essentially absent in corals exposed to low light, but their tissue content increases in proportion to photon flux densities beyond $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ (D'Angelo et al. 2008). As a consequence, the ectodermal tissue of the branch upperside of the purple morph (ESM Fig. S4) shows a strongly increased expression of *avalCP580* compared to the brown morph (Fig. 4). The branch undersides of both morphs are essentially CP free. Differences in the response to high irradiance were observed when the uppersides of the branches of the morphs were exposed to intense orange light ($1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$). Over the course of the light stress experiment, zooxanthellae of both morphs showed a clear reduction in the maximum quantum yield (F_v/F_m) after dark recovery. However, the decline in the upper branch sides was significantly greater in the brown morph with an almost 50 % reduction compared to 21 % in the purple morph (Mann–Whitney U test, $p < 0.05$). Similarly, the purple morph also showed a higher effective quantum yield at the end of the second light cycle than the brown morph. Only the branch upperside of the brown morph appeared visibly bleached by the end of the treatment (Fig. 4). When the shade-acclimated sides of the colonies, both without CP pigmentation, were exposed to the same conditions, the decline in the maximum quantum yield ($>75 \%$) was considerably larger than for the light-acclimated uppersides of the branches. The decrease was comparable between morphs (Mann–Whitney U test, $p = 0.421$) and consistently observed for the effective quantum yield. In both morphs, the treatment resulted in a bleached appearance of the shade-acclimated branch sides (Fig. 4).

Growth zone CP expression

Regenerating tissue of *A. polystoma* grown under higher light contained very low symbiont densities, whereas the new tissue of the low-light sample was fully colonised by zooxanthellae (Fig. 5). Under the higher light conditions, the tissue content of *apolCP584* contributed $\sim 6 \%$ to the total soluble protein of the high-light regenerating tissue, representing an ~ 30 -fold greater CP content than the new tissue grown under low light. The difference in CP concentration is reflected in the chlorophyll excitation spectra, with the higher CP content in the high-light tissue resulting in a relative reduction in chlorophyll excitation peaking at 582 nm. CP absorption was evident in the spectra of the high-light regenerating tissue. In agreement with our findings for *M. foliosa* (ESM Fig. S5), the lack of absorption by algal pigments in regenerating tissue under high light results in a high reflectance in the blue–green spectral range (ESM Fig. S6).

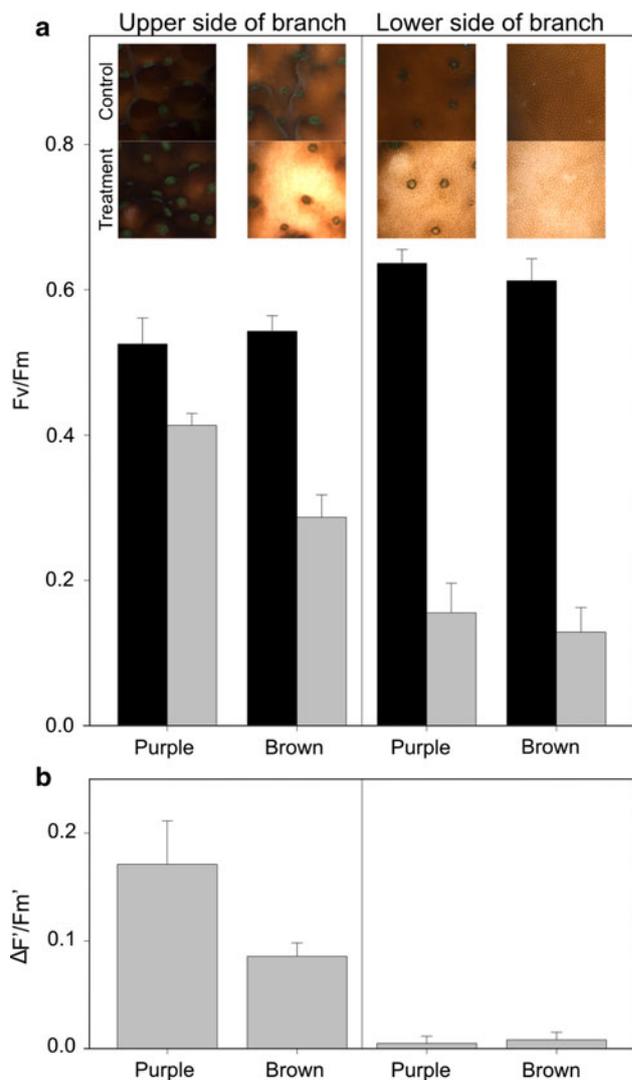


Fig. 4 The impacts of light stress on the photobiology of brown and purple *A. valida* morphs hosting subclade C3 symbionts. Comparison of maximum quantum yields before (*black bars*) and after (*grey bars*) the light stress treatment for light-acclimated upper (*left panel*) and shade-acclimated lower (*right panel*) tissues. The effective quantum yields are shown by the *light grey bars* and were performed prior to the end of the light stress treatment. The *error bars* show the \pm SD of 5 measurements. *Inset*: Photographs showing control and treatment corals

Discussion

The family of GFP-like proteins represents an important group of host pigments in reef-building corals and other anthozoans (Matz et al. 1999; Wiedenmann et al. 1999, 2000, 2004a; Salih et al. 2000; Dove et al. 2001; Alieva et al. 2008). It was proposed that these pigments exert a photoprotective function in corals and sea anemones by absorbing photons or redistributing them away from the major absorption bands of the photosynthetic pigments of the algal symbionts (Wiedenmann et al. 1999; Salih et al.

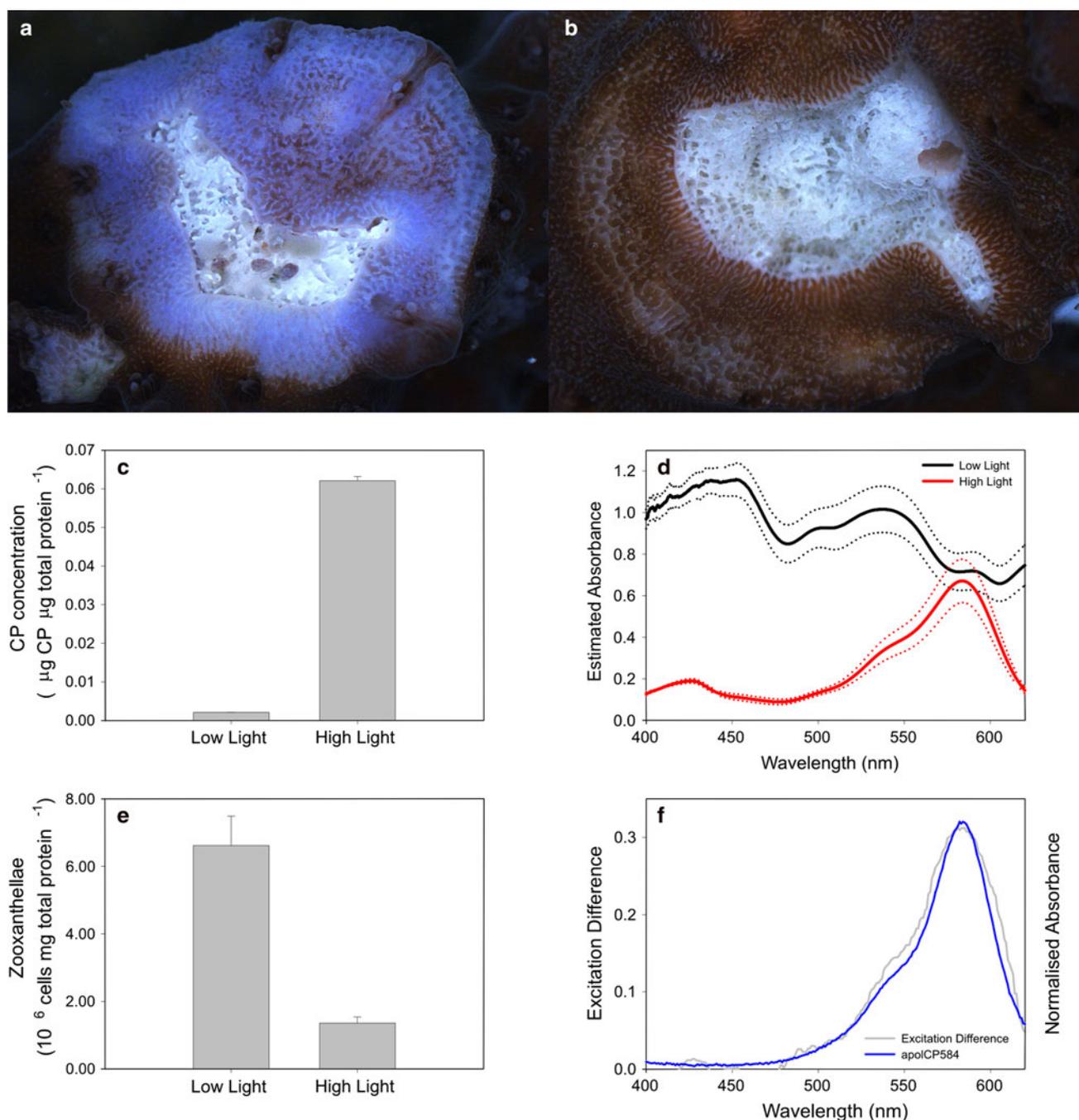


Fig. 5 Regulation of CP expression in regenerating tissue of *A. polystoma* under different light conditions. **a, b** Photographs of *A. polystoma* regeneration zones grown under high (**a**; $\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and low (**b**; $\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$) light. **c, e** CP (**c**) and zooxanthellae (**e**) content of regenerating tissue under the two light conditions. Error bars indicate the \pm SD of triplicate measurements.

d Mean estimated absorbance for the low- and high-light tissue. Dotted lines show the \pm SD of 5 independent measurements. **f** The excitation difference spectra of high- versus low-light regenerating tissue. The absorption spectrum of apolCP584 is shown for reference and is normalised to the height of the difference spectrum

2000; Dove et al. 2001). However, a general function of GFP-like proteins as photoprotectants has been controversial (Mazel et al. 2003; D'Angelo et al. 2008) and observations suggest functional heterogeneity among members of this large protein family (Alieva et al. 2008; D'Angelo

et al. 2008; Vogt et al. 2008). We tested the large group of non-fluorescent CPs for their potential to screen light that would be destined for zooxanthellae absorption, whether this screening provides relief from light stress and if screening is applicable to regions of low symbiont density.

CP screening

A previous study of 19 Caribbean coral species concluded that GFPs were not photoprotective as FPs did not significantly alter the chlorophyll excitation spectrum nor did they increase coral reflectance (Mazel et al. 2003). In contrast, we found that CPs have a pronounced effect on the algal chlorophyll excitation spectrum, with a clear decrease around the CP absorption maxima, both in vitro and *in hospite*.

The in vitro analyses provide a unique standpoint from which to monitor the CP effects on the chlorophyll excitation spectrum. Measurements were taken on identical populations of each clade of isolated zooxanthellae with different CP filters, and therefore, differences between the excitation spectra could be attributed solely to the CPs rather than differences in algal photobiology. While an in vitro experimental setup may not accurately represent the complex light environment of a coral's tissues (Kuhl et al. 1995; Enriquez et al. 2005), the close agreement in the degree of chlorophyll excitation reduction ($\sim 50\%$ at 580 nm) between our in vitro experiments (clade D, amilCP580) and an *A. valida* (clade D, avalCP580) colony from which the CP concentrations were calculated demonstrates that the results are indeed relevant to the *in hospite* CP function. Furthermore, the results show that screening occurs across the CP maximal range (562–604 nm). The measurable impact of CPs can be attributed to their high molar extinction coefficients, which tend to be significantly greater than those of their fluorescent counterparts, especially GFPs (Salih et al. 2006; Alieva et al. 2008; Nienhaus and Wiedenmann 2009).

The spectral analyses of zooxanthellae *in hospite* support the findings of the in vitro experiment, while furthering our knowledge of how CPs alter corals' optical properties. The excellent agreement between the excitation difference and CP absorption spectra clearly demonstrates the CP-mediated screening effect.

It is important to stress that screening in coral tissue is due to the physical separation of the two absorbing species, the zooxanthellae and the CPs. The light received by the gastrodermal zooxanthellae is directly dependent on the absorption and scattering occurring in the ectoderm that separates them from the external light field. The ectodermal CPs absorb light of certain wavelengths thereby reducing the amount of photons reaching the zooxanthellae in the endoderm. This screening results in a reduction in the chlorophyll excitation similar to the screening by epidermal anthocyanins in higher plants (Liakopoulos et al. 2006; Merzlyak et al. 2008).

CPs absorb photons in a spectral range that lies mostly outside the major absorption bands of photosynthetic pigments (D'Angelo et al. 2008; Dove et al. 2008).

Therefore, it has been argued that any photoprotection afforded by CPs is likely to be indirect, for instance, by a blue-light enrichment of the internal light field that promotes chlorophyll retention, increased antioxidant pools and expression of photoprotective Early Light-inducible Proteins (ELiPs) (Dove et al. 2008). While these assertions are likely valid, our results demonstrate that the role of CP absorption in photoprotection is also direct, particularly as the in vitro experiment demonstrated that total chlorophyll excitation between 400 and 620 nm could be reduced by up to 18%. The zooxanthellae chlorophyll excitation spectra determined for five coral species clearly show that light-use efficiency is less spectrally discriminant than absorption spectra of the pigments in dilute solutions would suggest. These results are consistent with photosynthetic action-spectra measured with fluorescence (Lesser et al. 2000; Mazel et al. 2003) and oxygen sensors (Kuhl et al. 1995) where the intensity parameter is the incident irradiance. This effect results from strong scattering and pigment-packaging occurring at the level of the symbionts inside the coral tissue (Kirk 1994; Stambler and Dubinsky 2005). As a result, the contribution of wavelengths outside the major pigment absorption bands to the excitation of the symbionts' chlorophyll fluorescence is substantial.

Photosynthetic antennae may not be the only site responsible for photodamage as the manganese complex of the oxygen evolving complex (OEC) has also been implicated in photoinhibition (Hakala et al. 2005; Baird et al. 2009; Takahashi et al. 2010). Although predominantly absorbing in the UV region of the spectrum, high photon fluxes throughout the visible spectrum can cause damage to the OEC (Hakala et al. 2005). Indeed, Takahashi et al. (2010) found that the yellow–orange region of the visible spectrum is the most damaging for higher plants despite low absorption in this spectral range by photosynthetic pigments. In fact, yellow-orange light remains an important spectral component in shallow water (Kirk 1994). Dustan (1982) found that even at 3 m depth, a Caribbean reef received more light at 567 nm than in blue wavelengths <500 nm. As other existing holobiont photoprotective mechanisms, such as MAA accumulation, act outside the yellow–orange range, the CPs in shallow-water corals might provide protection in a vulnerable spectral window, functioning in a similar manner as anthocyanins in higher plants (Merzlyak et al. 2008). Finally, important physiological processes in corals, including regulation of genes encoding GFP-like proteins, are controlled by blue light (Levy 2003; Levy et al. 2006; D'Angelo et al. 2008; Kaniewska et al. 2009). Hence, the removal of photons in the longer wavelength range appears to be a sensible strategy to avoid interference between photoprotection and blue-light-regulated processes.

CP screening correlates with reduced photodamage

We assessed the potential of screening to afford relief from light stress by subjecting two colour morphs of *A. valida* to high photon fluxes of yellow–orange light ($1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$) and measuring the reduction in the maximum quantum yield in response to light stress after 12 h dark recovery. This value is indicative of photodamage rather than dynamic photoinhibition as the dynamic processes should relax within ~ 3 h (Gorbunov et al. 2001). The method has been used in previous studies and the results correlate with changes in other photodamage biomarkers such as D1 concentration and symbiont cell viability (Warner et al. 1999; Gorbunov et al. 2001; Franklin et al. 2004). We found that the CP-containing tissues of the branch uppersides of the purple morph of *A. valida* suffered less photodamage under high-light stress compared to the brown morph. In contrast, comparable damage was observed when the CP-free tissue of the branch undersides of both morphs was subjected to the light stress treatment. Hence, the finding that zooxanthellae in tissues containing high CP concentrations show reduced damage under the impact of light stress strongly supports an ecologically relevant photoprotective function of CPs. It is important to stress, however, that CP expression is certainly not vital for shallow-water corals, otherwise it would be ubiquitous. Instead, they are likely to represent a strategy for extending the ecological niche of certain species. It is established that zooxanthellae have mechanisms that are effective in protecting the photosynthetic apparatus under supraoptimal irradiances (Gorbunov et al. 2001), and therefore, it has been questioned whether there is a need for additional host-based photoprotectants (Mazel et al. 2003). However, there is evidence that under shallow-water light conditions on a cloud-free day and under thermal stress, the symbionts' capacity for photoprotection can be exceeded and photodamage can occur (Iglesias-Prieto et al. 1992; Warner et al. 1999; Gorbunov et al. 2001). We propose that under these conditions, CP expression may help the coral to minimise light stress. Moreover, the CPs could additionally act to promote survival during occasional periods of extraordinary stress.

A photoprotective role for GFPs has been questioned because bathymetric studies of the GFP content of *Montastraea faveolata* and *M. cavernosa* showed no significant correlation with depth and other species lacked the light-dependent regulation of GFP-like proteins that would be expected for a typical photoprotectant (Mazel et al. 2003; Wiedenmann et al. 2004b; Leutenegger et al. 2007a; Vogt et al. 2008). However, D'Angelo et al. (2008) demonstrated a light intensity–controlled production of FPs and CPs in shallow-water corals. In agreement with that study, we saw a pronounced accumulation of CPs in the upper side of

branches in field samples and in experimental corals grown under controlled light conditions. Apparently, not all corals demonstrate a light-regulated expression of GFP-like proteins (Leutenegger et al. 2007a). However, the high-light-induced production of CPs supports a photoprotective function and helps to explain the depth-dependent decrease in morphs with pronounced CP colouration observed in a previous study (Salih et al. 2006). The co-existence of CP-containing and CP-free morphs in shallow water supports the assertion that protection provided by the pigments is not vital but potentially offers more subtle benefits enabling corals to acclimate to a broader range of conditions in habitats characterised by a highly dynamic light field.

Screening by CPs in regions of low symbiont density

A constrained localisation of FPs to structural features, such as skeletal ridges, is typically found where the zooxanthellae concentration is lowest (Mazel et al. 2003). It was concluded that the physical segregation from the algae would preclude FPs from playing a role in symbiont photobiology (Mazel et al. 2003). Moreover, the high-level CP expression in coral growth zones that are essentially free from zooxanthellae (D'Angelo et al. 2012) called for further analyses of the function of GFP-like proteins in these regions. Here, we investigated whether the presence of CPs could afford screening to growth regions such as coral tips or margins, regenerating colony parts or areas in which an epibiont infestation stimulated a growth-mediated immune response (D'Angelo et al. 2012). In the regenerating tissue of *A. polystoma* grown under low light, CP expression was largely absent, whereas high-light colonies amassed a ~ 30 -fold increase in CP concentration, confirming a light-driven upregulation of CP expression (D'Angelo et al. 2008). CP localisation to growing areas devoid of zooxanthellae can be explained by the high internal light fluxes in the blue–green spectral range that result from the lack of absorption of incident light by the photosynthetic pigments of the symbiont and its reflection by the coral skeleton, potentially amplified by multiple scattering (Enriquez et al. 2005). The comparison of reflectance spectra of tissue areas with and without zooxanthellae suggests a 5–7-fold increase in internal fluxes of blue–green light in growing regions of the colony. A comparable increase in light intensity was proven to stimulate an enhanced production of GFP-like proteins (D'Angelo et al. 2008).

Once the symbiont population becomes established, CP expression decreases due to the reduction of internal blue-light fluxes. Consequently, the light-driven upregulation and the transient appearance of CPs in the growth zones could enable certain corals to provide screening to parts of the colony of greatest need, without compromising the

overall productivity. Furthermore, considering that axial polyp development is blue-light dependent (Kaniewska et al. 2009), the absorption characteristics of the CPs in growth zones are ideally suited to provide screening without interfering with blue-light-regulated growth processes.

In agreement with our spectral analyses of established tissues, CP expression in regenerating tissues is clearly capable of screening the symbionts. We postulate that the presence of CPs in growth zones assists in the colonisation of new coral tissue of pigmented morphs by providing photo-protection from the high internal light fluxes associated with multiple scattering in the coral skeleton (Enriquez et al. 2005; Terán et al. 2010).

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Screening by coral green fluorescent protein (GFP)-like chromoproteins supports a role in photoprotection of zooxanthellae

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Electronic Supplementary Materials

ESM Methods

Fluorescence spectroscopy

The excitation spectrum of PSII emission at 680nm was measured from 400-620nm with excitation and emission slits widths of 10nm and 5nm, respectively. We conducted initial fluorescence excitation and emission measurements on all studied species to ensure that emission at 680nm was solely due to chlorophyll emission (ESM Fig. S7) with no interference from other fluorophores such as red fluorescent proteins. For comparison of coral tissues with different pigmentation, the chlorophyll excitation spectra were normalised to the 460nm peak under the assumption that excitation at 460nm is unaffected by host pigments (Mazel et al. 2003).

Excitation difference spectra were calculated to explore the spectral nature of the pigments driving the observed changes in chlorophyll excitation spectra. Here, the chlorophyll excitation of CP pigmented tissue was subtracted from the non-CP pigmented tissue and the difference was normalised to the excitation at that wavelength of the brown morph. As an example, an excitation difference value of 0.5 would represent 50% lower excitation of chlorophyll in the CP pigmented tissue compared to the non-CP pigmented tissue at that specific wavelength. The excitation difference spectra peaks were consistent across the chlorophyll emission spectrum (ESM Fig. S7c).

CP quantification

In order to enable quantification of aValCP580 and aPolCP582, the molar extinction coefficients of these CPs were determined using the alkaline denaturation method (Kredel et al. 2008). As the amino acid sequence for these CPs was unknown, the molecular weight of CPs was estimated by averaging the molecular weights of five acroporid CPs (GenBank: AAU06853.1, AAU06856.1, AAU06855.1, AAU06854.1, ACH089425.1) (Alieva et al. 2008; D'Angelo et al. 2008) calculated with Lasergene's Protean software (DNASTAR, USA). Considering the

low standard deviation ($\pm 0.23\%$) of the individual molecular weights from the mean (25 kDa), the estimates can be expected to be representative.

The CP concentration in clarified protein extracts from the purple morph of *A. valida*, and from the growth zones of *A. polystoma* under two light conditions was quantified by spectral unmixing using the method of least squares (Settle and Drake 1993; Hedley et al. 2004). This study uses absorption spectra in the unmixing as, according to Beer's Law, the absorption of a mixture can be considered to be the additive sum of the weighted absorption coefficients of the components within the mixture (Kirk 1994). The endmember spectra used in the two-endmember unmixings were the purified CP spectrum and the spectrum of a clarified protein extract taken from an independent tissue sample from the respective species (*A. valida* – brown morph; *A. polystoma* – low light). All spectral unmixings were performed on clarified tissue extracts with absorption readings less than 1.0 to ensure linearity. CP concentrations were calculated using the extinction coefficients and were normalised to the total soluble protein concentrations (BCA assay, Pierce) and/or the coral surface.

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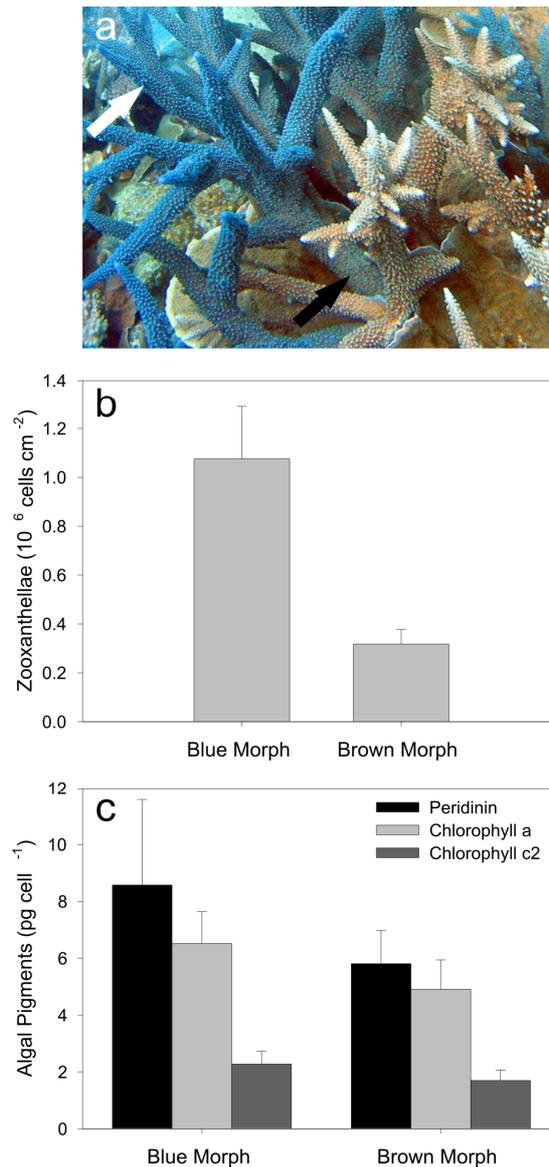
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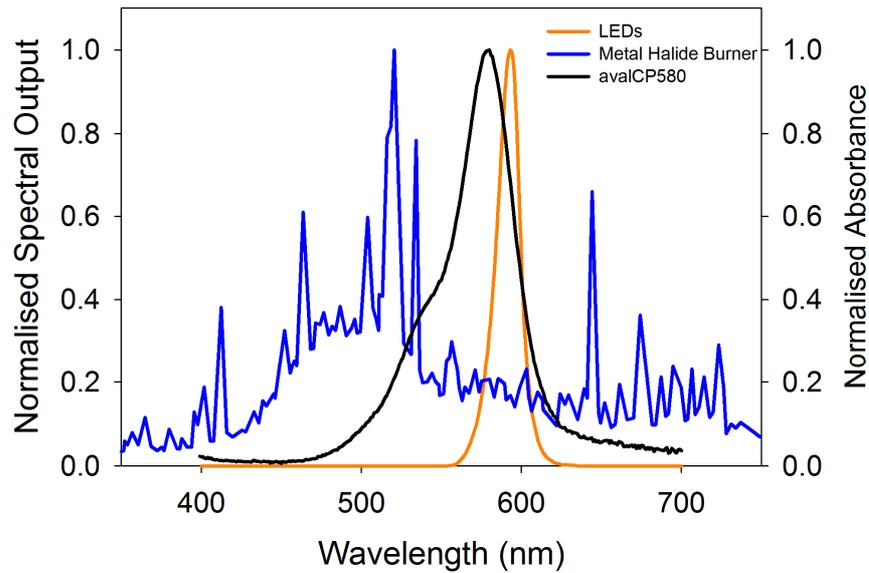
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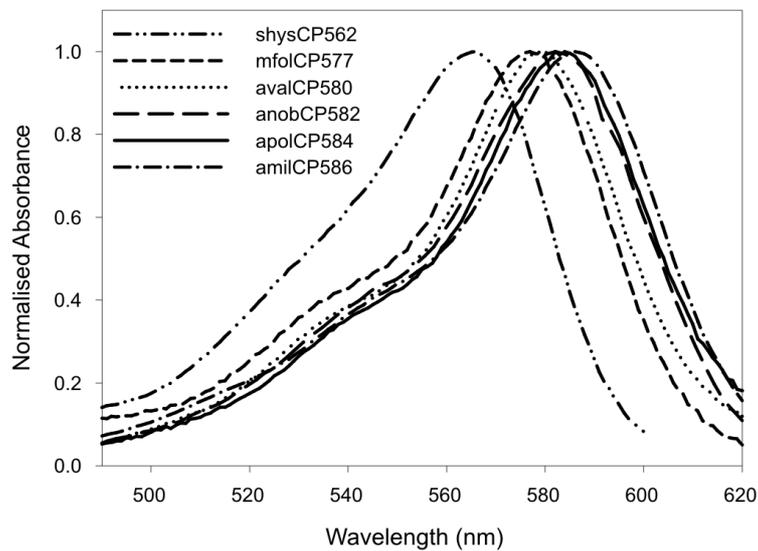
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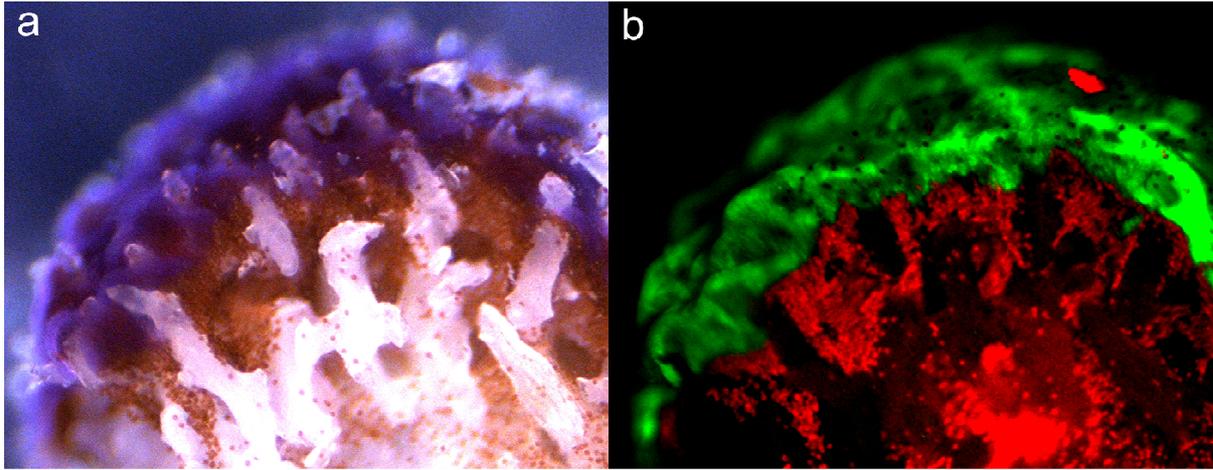
ESM Figure S1. Analysis of blue and brown morphs of *Acropora nobilis* from the Great Barrier Reef. (a) Photograph of the two morphs side-by-side on the reef. The white arrow indicates a light exposed branch whereas the dark arrow shows a branch that is subject to self shading within the blue morph colony. (b, c) Mean zooxanthellae densities (b) and pigment concentrations (c) from five replicate colonies collected at 1.5 m. Error bars indicate the \pm SD of the mean.



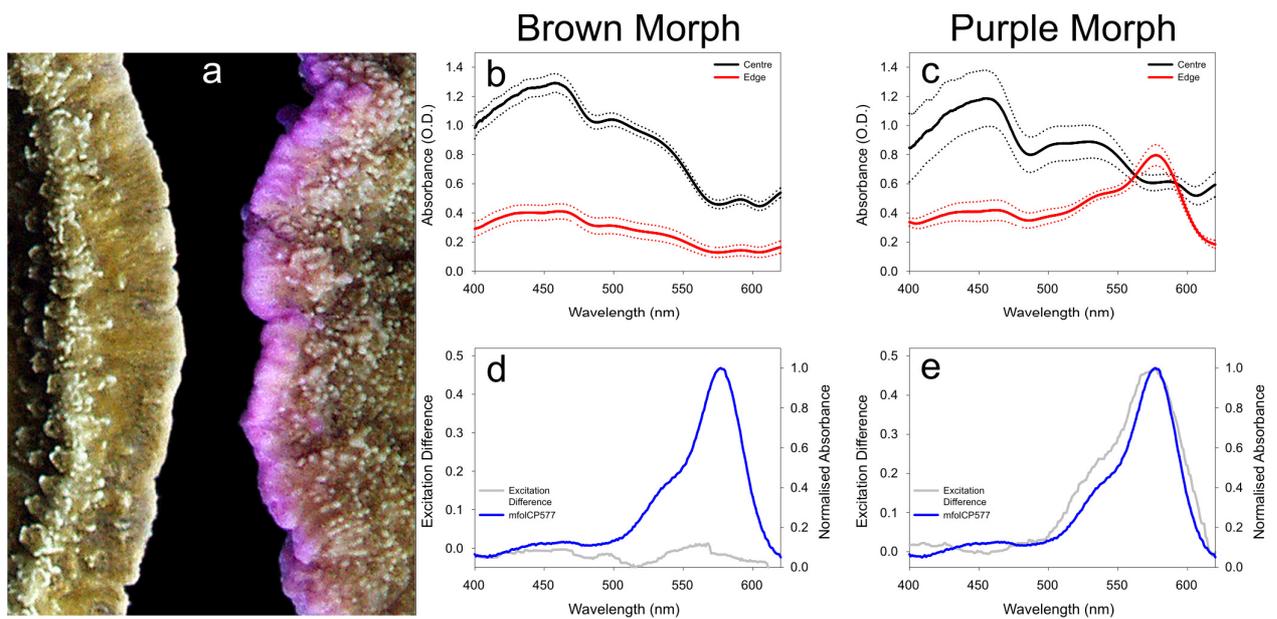
ESM Figure S2. Optical properties of the light sources used in this study. The normalised spectral output of the metal halide burners used for growing the aquarium corals and the amber LEDs used for the high irradiance experiments. The absorption spectrum for avalCP580 is shown to demonstrate the overlap between the LED output and the absorption of the CP.



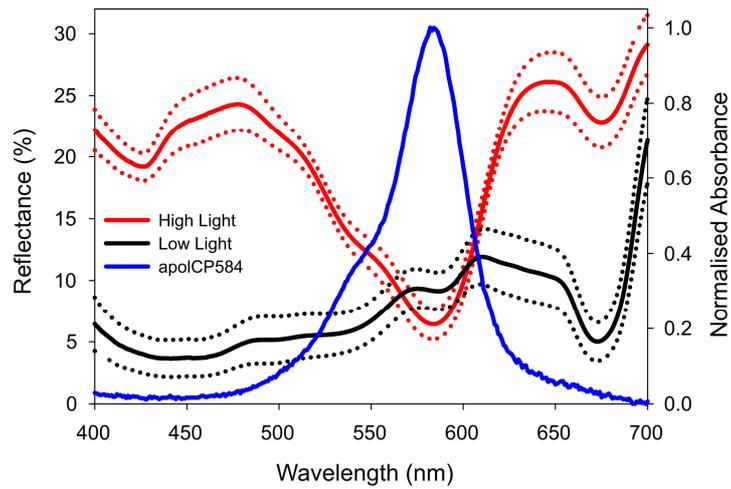
ESM Figure S3. Absorption spectra of CPs isolated and purified from the corals *Seriatopora hystrix* (shysCP562), *Montipora foliosa* (mfolCP577), *Acropora valida* (avalCP580), *Acropora nobilis* (anobCP582), *Acropora polystoma* (apolCP584) and *Acropora millepora* (amilCP586). Spectra are normalised to a peak height of 1.0.



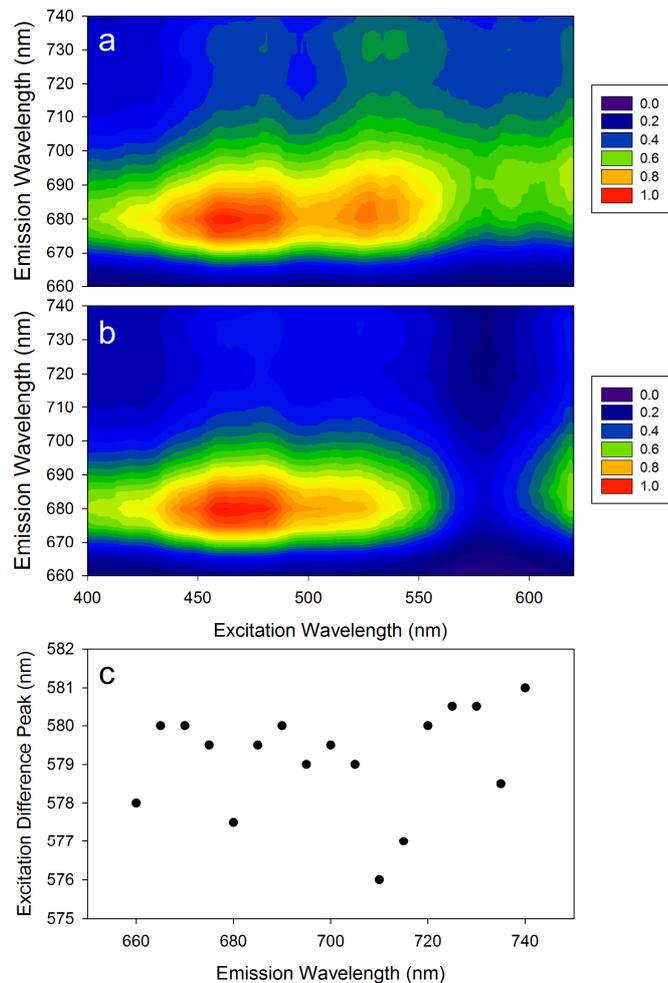
ESM Figure S4. Localisation of *avalCP580* in the purple morph of *Acropora valida*. (a) Micrograph of a cross section of an *A. valida* tip showing the localisation of the purple chromoprotein. (b) Fluorescent image of the corresponding cross section showing the presence of ectodermal green fluorescent proteins (green) and chlorophyll fluorescence from the zooxanthellae (red).



ESM Figure S5. Characterisation of chromoproteins in growth regions. (a) Photograph of two *Montipora foliosa* morphs expressing (right) and not expressing (left) chromoproteins in the colony margins. (b, c) Mean approximated absorbance spectra of the brown (b) and purple morphs (c) at the centre and growth margins of the colonies. Dotted lines indicate the \pm SD of 5 independent measurements. (d, e) Excitation difference spectra for the brown (d) and purple (e) morphs. The absorption spectra of *mfolCP577* are shown for reference.



ESM Figure S6. Reflectance spectra of regenerating zones in *Acropora polystoma*. The solid lines show the mean reflectance of the high and low light regeneration zones with the standard deviations of 5 measurements shown by the dotted lines. The absorption spectrum of apolCP584 is shown for comparison.



ESM Figure S7. Excitation and emission properties of the brown and purple colour morphs of *Acropora valida*. (a, b) Excitation-emission plot for the brown (a) and purple (b) morphs. The excitation spectra are sampled at 1 nm intervals and the emission spectra are sampled every 5 nm. (c) Difference spectrum peak values across the chlorophyll emission range. The mean peak wavelength of the difference spectrum is 579 nm with a standard deviation of 1 nm and the respective CP absorption peak is at 580 nm.