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Contributions of host and symbiont pigments to the coloration of reef corals

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For a variety of coral species, we have studied the molecular origin of their coloration to assess the contributions of host and symbiont pigments. For the corals *Catalaphyllia jardinei* and an orange-emitting color morph of *Lobophyllia hemprichii*, the pigments belong to a particular class of green fluorescent protein-like proteins that change their color from green to red upon irradiation with ~400 nm light. The optical absorption and emission properties of these proteins were characterized in detail. Their spectra were found to be similar to those of phycoerythrin from cyanobacterial symbionts. To unambiguously determine the molecular origin of the coloration, we performed immunochemical studies using double diffusion in gel analysis on tissue extracts, including also a third coral species, *Montastrea cavernosa*, which allowed us to attribute the red fluorescent coloration to green-to-red photoconvertible fluorescent proteins. The red fluorescent proteins are localized mainly in the ectodermal tissue and contribute up to 7.0% of the total soluble cellular proteins in these species. Distinct spatial distributions of green and cyan fluorescent proteins were observed for the tissues of *M. cavernosa*. This observation may suggest that differently colored green fluorescent protein-like proteins have different, specific functions. In addition to green fluorescent protein-like proteins, the pigments of zooxanthellae have a strong effect on the visual appearance of the latter species.

Reef-building corals are famous for their spectacular colors, ranging from blue and green to yellow, pink, orange and red. Green fluorescent protein (GFP)-like proteins contribute to this coloration in a major way. They were initially discovered in nonbioluminescent, zooxanthellate anthozoa, including actiniaria, zoantharia, corallimorpharia and stolonifera [1–4], and subsequently recognized as major color determinants of hermatypic reef corals [5–7] and also of azooxanthellate anthozoans [8].

In addition to GFP-like proteins from the anthozoa, the presence of symbionts also contributes to reef coloration. The brownish tones of cnidarians may arise from symbiotic algae of the genus *Symbiodinium*, the

Abbreviations
cjarRFP, *Catalaphyllia jardinei* red fluorescent protein; EosFP, Eos fluorescent protein; FP, fluorescent protein; GFP, green fluorescent protein; lhemOFP, *Lobophyllia hemprichii* orange fluorescent protein; mcavRFP, *Montastrea cavernosa* red fluorescent protein; rPE, phycoerythrin from the red alga *Fauchea* sp.; scubRFP, *Scolymia cubensis* red fluorescent protein.

so-called zooxanthellae, which enable their coral hosts to thrive in oligotrophic waters by supplying photosynthetic products such as carbohydrates and amino acids. Temperate cnidarians contain mostly a single type of zooxanthella, designated as 'temperate A' [9,10]. In contrast, tropical corals can host different genotypes, the so-called 'clades' of *Symbiodinium* [11]. The content of zooxanthellae clades may vary within a species and even within a single colony [12,13]. Corals have been endangered by episodes of fatal losses of their symbionts [14,15]. The process was termed 'coral bleaching', because the reduction in zooxanthellae pigments rendered the animal color whitish.

Recently, the presence of additional, cyanobacterial symbionts was suggested for *Montastrea cavernosa*, which may contribute to coral nutrition by 'fertilizing' the zooxanthellae with fixed nitrogen [16]. The orange fluorescence of *M. cavernosa*, peaking at 580 nm, was attributed to phycoerythrin of the cyanobacterial symbiont, whereas previous reports had shown that the color mainly arises from the GFP-like protein *M. cavernosa* red fluorescent protein (mcavRFP), the emission of which peaks at 582 nm [6,17,18]. This protein belongs to a class of fluorescent proteins that change their emission colors irreversibly from green to red upon irradiation with light of wavelengths around 400 nm [17–20]. The color change in this protein class arises from a photoinduced extension of the delocalized π-electron system of the chromophore, which is accompanied by a break in the protein backbone adjacent to the chromophore [21,22]. So far, green-to-red photoconverting fluorescent proteins have been isolated from six anthozoan species [17,19,20,22].

The widespread abundance and color diversity of GFP-like proteins in anthozoa suggest specific biological functions, which have not yet been unambiguously identified and are still controversial [23–27]. GFP-like proteins have been suggested to exert a photoprotective function or to render the internal light spectrum favorable for zooxanthellae photosynthesis [28–31]. However, the nature of the photoprotective mechanism has remained elusive. Further studies of the functions of GFP-like proteins are necessary for assessing the adaptability of coral ecosystems in times of global climate change. Specific knowledge of coral pigmentation is a prerequisite for these studies, and therefore we have analyzed in detail the origin of red fluorescent coloration of Faviina and Meandriina corals.

Results and Discussion

GFP-like proteins contribute in a major way to the coloration of *M. cavernosa*. The red fluorescent morph showed high-level transcription of the green-to-red photoconvertible protein mcavRFP. In the green morph, the transcript of a cyan fluorescent homolog was most abundant; a GFP-like protein almost identical to mcavRFP was transcribed only to a minor extent [6].

Fluorescence similar to that seen in the red morph of *M. cavernosa* was also observed in the tentacle tips of *Catalaphyllia jardinei*, with a fluorescence emission peak at ~581 nm, and we also noticed a peculiar orange fluorescence with an emission maximum at 572 nm in a particular colony of *Lobophyllia hemprichii*. To investigate whether the pigments responsible for the coloration are GFP-like proteins or other pigments such as phycoerythrin, we constructed cDNA libraries of two individuals of the two species. We amplified cDNAs coding for two GFPs from these cDNA libraries. The protein from *C. jardinei* shares 87.6% identical residues with Kaede from *Trachyphyllia geoffroyi* [19]. With 67.3% identical residues, the protein from *L. hemprichii* shows a slightly higher similarity to mcavRFP than to Eos fluorescent protein (EosFP) (64.8%) from another color morph of *L. hemprichii* (supplementary Fig. S1). The similarity of the proteins is also reflected in the identical fluorescence spectra, peaking approximately at 506 nm (excitation) and 516 nm (emission) (Fig. 1). These two novel proteins share a histidine with green-to-red photoconverting fluorescent proteins (FPs) as the first residue of the chromophore tripeptide (supplementary Fig. S1). Indeed, they also change their emission color from green to red upon irradiation with light of ~400 nm (Fig. 1). Photoconversion is accompanied by the characteristic cleavage of the polypeptide backbone, yielding subunits with molecular masses around 20 kDa and 8 kDa (Fig. 2). The fluorescence maxima of the red-shifted states are identical to those determined from the animal tissues. This observation implies that the unusual orange fluorescence observed in *L. hemprichii* is also derived from a green-to-red photoconverting protein. According to the taxonomic origin and the emission color, the novel proteins were named cjarRFP (*C. jardinei* red fluorescent protein) and lhemOFP (*L. hemprichii* orange fluorescent protein).

Both proteins form tetramers, as deduced from their elution behavior in size exclusion chromatography (data not shown) [20]. The spectral properties of the red form of cjarRFP are surprisingly similar to those of EosFP and other known representatives of green-to-red photoconvertible proteins (Table 1). As shown in Fig. 1 and Table 1, the red-shifted state of lhemOFP, however, shows striking differences to that of EosFP: (a) its emission maximum is localized at 574 nm and therefore shifted by 7 nm to the blue; (b) its excitation

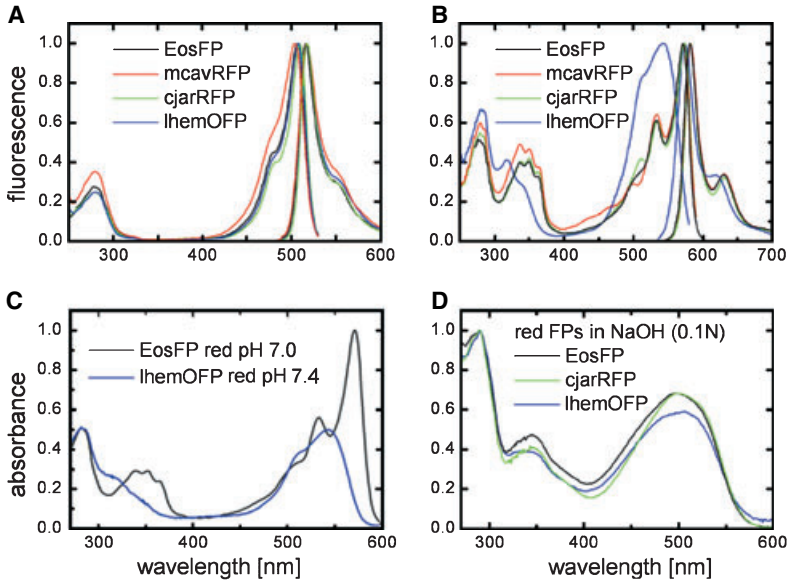

Fig. 1. Comparison of spectral properties of the green-to-red photoconverting proteins cjarRFP, lhemOFP, mcavRFP and EosFP. (A) Excitation and emission spectra of the green fluorescent states are essentially identical. (B) Both excitation and emission spectra of the red state of lhemOFP are blue-shifted in comparison to cjarRFP, mcavRFP and EosFP. (C) Absorption spectra of the red chromophores of lhemOFP and EosFP. (D) The absorption maxima of alkaline-denatured proteins peaking around 500 nm are indicative of identical chromophore structures.

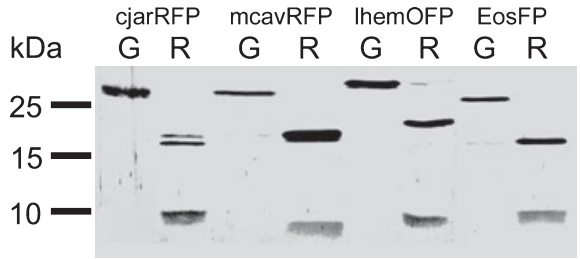

Fig. 2. Gel analysis of the purified green-to-red photoconverting proteins cjarRFP, lhemOFP, mcavRFP and EosFP. Both green (G) and red states (R) of the proteins were separated by SDS/PAGE. (A) Photoconversion is accompanied by dissociation into subunits of ~20 and ~8 kDa.

maximum is at 543 nm, and consequently, its Stokes shift of 31 nm is more than twice as large as for the other photoconvertible proteins; and (c) its excitation spectrum is rather broad, and the vibronic band at 533 nm is not clearly visible.

For EosFP, X-ray crystallography has revealed that the entire structure of the chromophore and the protein scaffold remains essentially unperturbed upon green-to-red photoconversion [21]. Even the network of hydrogen bonds is left unaltered by the β-elimination reaction that leads to photochemical modification of the chromophore and cleavage of the peptide backbone between Nα and Cα of His62.

Homology modeling suggests that the hydrogen-bonding residues surrounding the EosFP chromophore are also present in lhemOFP (supplementary Fig. S1). The conserved chromophore environment of lhemOFP explains the essentially identical spectral properties of the green fluorescent state (Fig. 1). The absorption maximum of the alkaline-denatured photoconverted protein peaks around 500 nm and is identical to those of cjarRFP, mcavRFP and EosFP. The molar extinction coefficient of the alkaline-denatured chromophores was about 28 000 $M^{-1}\cdot cm^{-1}$ in all cases. This result suggests that the 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-

Table 1. Spectral properties of green-to-red photoconvertible fluorescent proteins.

	$\lambda_{max\ green}$ (nm) ex/em	$\varepsilon_{mol\ green}$ ($M^{-1}\cdot cm^{-1}$)[a]	QY_{green}	τ_{green} (ns)	$\lambda_{max\ red}$ (nm) ex/em	$\varepsilon_{mol\ red}$ ($M^{-1}\cdot cm^{-1}$)[a]	QY_{red}	τ_{red} (ns)
Kaede[b]	508/518	98 800	0.80	–	572/580	60 400	0.33	–
EosFP[c]	506/516	72 000	0.70 ± 0.02	2.9 ± 0.1	571/581	41 000	0.55 ± 0.03	3.6 ± 0.1
mcavRFP	504/517	53 100	0.60 ± 0.03	3.6 ± 0.3	571/582	60 000	0.6 ± 0.03	4.0 ± 0.1
cjarRFP	509/517	105 000	0.55 ± 0.03	2.2 ± 0.2	573/582	55 800	0.56 ± 0.03	3.0 ± 0.1
lhemOFP	507/517	94 200	0.58 ± 0.02	3.1 ± 0.2	543/574	36 300	0.65 ± 0.03	3.5 ± 0.1

[a] Chromophores may exist in a pH-dependent equilibrium of protonation states [37]. The molar extinction coefficients in this table were calculated from the peak absorptions of the bands at pH 7.0 to provide a measure of coloration. [b] Data from [19]. [c] Data from [41].

4-(*p*-hydroxybenzylidene)-5-imidazolinone structure is the common chromophore of the red forms of green-to-red photoconvertible proteins (Fig. 1) [21]. Consequently, the unusual characteristics of the red-shifted fluorescence of lhemOFP may indicate a spatial rearrangement of the chromophore or its surroundings upon photoconversion, which may be mediated by more distant residues. Possibly, the conjugated π-electron system does not extend as far into the imidazolyl group of the chromophore as in other green-to-red photoconverting proteins, due to a noncoplanar arrangement of the imidazole moiety with the rest of the chromophore. Effects of tetramer interface mutations on the optical properties were observed earlier for EosFP [36]. The crystal structure of lhemOFP is expected to yield further insights into the chemical nature of its red chromophore.

With 3.5 ± 0.1 ns, the fluorescence lifetime of lhemOFP is slightly shorter than the 4.0 ± 0.1 ns determined for mcavRFP (Table 1). The latter value is similar to the fluorescence lifetime of 3.93 ns attributed to cyanobacterial phycoerythrin from *M. cavernosa* [16]. With values ranging from ~0.04 ns to 1.74 ns, considerably shorter fluorescence lifetimes were reported for phycoerythrins from other cyanobacteria and red algae [37,38]. Our characterization of the fluorescent pigments of *C. jardinei* and the orange morph of *L. hemprichii* further emphasizes a major role of GFP-like proteins in the coloration of scleractinian corals.

To unambiguously distinguish between the contributions of GFP-like proteins and phycoerythrin to coral coloration [16], we applied an immunochemical approach. This novel method is based on double diffusion in gel analysis, and uses the intrinsic fluorescence of test compounds for the detection of cross-reactivity with an antiserum [35]. If the antigens, GFP-like proteins or phycoerythrin from the red alga *Fauchea* sp. (rPE) or the nonsymbiotic cyanobacterium *Lyngbya* sp. are crosslinked by the antibodies, a fluorescent precipitate will form. In contrast, cross-reactivity with other proteins will yield a whitish, nonfluorescent precipitate. First, we tested tissue extracts from the red fluorescent morphs of *L. hemprichii* and *M. cavernosa*, the orange morph of *L. hemprichii*, and extracts of the red alga *Fauchea* sp. and the cyanobacterium *Lyngbya* sp. for their cross-reactivity with an antiserum raised against B-phycoerythrin from the red alga *Porphoridium cruentum* [39] (Fig. 3). This antiserum was used to demonstrate the presence of phycoerythrin in *M. cavernosa* [16]. Under daylight, whitish bands in the agarose gel indicated cross-reactivity of the antiserum with the extracts of *M. cavernosa* and *Fauchea*

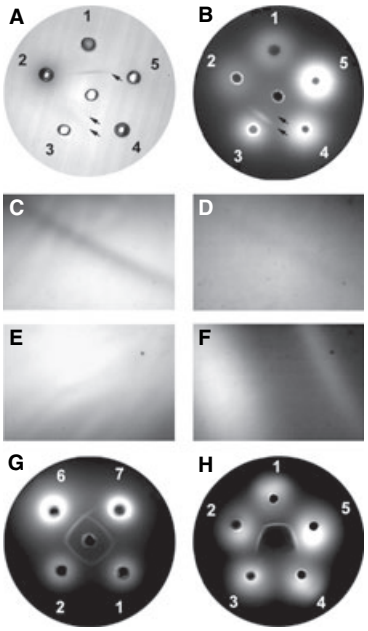

Fig. 3. Double diffusion in gel analysis of tissue extracts of the red morph of *M. cavernosa* (1), the red morph of *L. hemprichii* (2), the red alga *Fauchea* sp. (3), nonsymbiotic cyanobacteria (4), the orange morph of *L. hemprichii* (5) and the purified recombinant proteins mcavRFP (6) and EosFP (7). (A) Daylight photograph of an agarose plate with diffusing coral, algal and bacterial extracts. Precipitates are highlighted by arrows and indicate the cross-reactivity of the antiserum raised against phycoerythrin with proteins of *M. cavernosa* and *Fauchea* sp. (B) Fluorescence photograph of the same agarose plate. Samples were excited with blue light, and fluorescence was photographed through a red filter glass (long pass 550 nm). The extract of *Fauchea* sp. shows one dominant and one faint fluorescent precipitate (arrows), whereas the fluorescent pigments from cyanobacterial and coral extracts are freely diffusing. (C) Bright-field microscopy image of the precipitate of the extract of *M. cavernosa*. (D) Fluorescence micrograph of the *M. cavernosa* extract obtained using a tetramethylrhodamine B isothiocyanate filter set. Upon excitation with green light, the red fluorescence of the freely diffusing pigment is visible, whereas no red fluorescence of the precipitate could be detected. (E) Bright-field microscopy image of the area of the agarose plate containing the precipitates of the red alga extract. Precipitates could not be imaged under these conditions. (F) Fluorescence micrograph of the same region of interest obtained using a tetramethylrhodamine B isothiocyanate filter set. Intense red fluorescence is emitted from the precipitates and reveals the presence of rPE in the red algal extracts. (G–H) Agarose plates excited with blue light and photographed using a red filter glass (long pass 550 nm). Fluorescent bands indicate the cross-reactivity of the antiserum against EosFP/mcavRFP with the purified antigens used for immunization (6–7) and the red fluorescent pigments of the red morphs of *M. cavernosa* (1) and *L. hemprichii* (2). The fluorescent pigments from extracts of the orange morph of *L. hemprichii* (5) also precipitate in the presence of the anti-EosFP/mcavRFP serum. The free diffusion of fluorescent molecules in the extracts of red alga and cyanobacteria shows that the serum does not react with phycoerythrin, either from *Fauchea* sp. (3) or from *Lyngbya* sp. (4).

sp. (Fig. 3A). Under excitation with blue light, the precipitates of *Fauchea* sp. showed strong orange fluorescence, indicating the cross-reaction of the serum with rPE (Fig. 3B). In contrast, the band of *M. cavernosa* was nonfluorescent, and free diffusion of the fluorescent pigments was visible. Free diffusion of fluorescent molecules could also be observed for the extracts from *L. hemprichii* and cyanobacteria (Fig. 3B). An analysis of the precipitates under the fluorescence microscope confirmed the findings of the macroscopic inspection (Fig. 3C–F). These results clearly demonstrate that the antiserum raised against B-phycoerythrin [39] does indeed display cross-reactivity with a protein from *M. cavernosa*; however, this protein is not fluorescent. Also, an antiserum against red algal phycoerythrin (from Biomeda, Foster City, USA) did not show cross-reactivity with the fluorescent pigments from the coral extracts (data not shown). Therefore, the fluorescent color of this coral cannot be attributed to a fluorescent phycoerythrin.

However, photosynthetic pigments from symbionts can indeed affect the coloration of *M. cavernosa* (supplementary Fig. S2). The tissue content of the zooxanthellae pigments chlorophyll *a* and peridinin was increased $\sim$5-fold in animals kept under a photon flux of 100 μmol $m^{-2} \cdot s^{-1}$ in comparison to colonies grown under four-fold higher light intensity, resulting in a visually darker appearance.

To prove that the freely diffusing red fluorescent pigments are indeed GFP-like proteins, we raised an antiserum against recombinantly produced EosFP and mcavRFP. As demonstrated by western blot analysis, the antiserum specifically recognizes the novel photoconverting proteins cjarRFP and lhemOFP in addition to the proteins used for immunization (supplementary Fig. S3). Subsequently, the antiserum was applied in the double diffusion test against recombinant EosFP and mcavRFP and tissue extracts of *M. cavernosa* and the red morph of *L. hemprichii* (Fig. 3G). A strongly fluorescent precipitate formed in all cases. Cross-reactivity with the fluorescent pigment was also detected for tissue extracts of the orange morph of *L. hemprichii* (Fig. 3H). Conversely, immunoreactivity was absent for the red algal and cyanobacterial tissue extracts. We conclude, therefore, that the fluorescent coloration of *M. cavernosa* and *L. hemprichii* is due to GFP-like proteins and not caused by a fluorescent phycoerythrin. The contribution of mcavRFP, EosFP and lhemOFP to the total content of soluble proteins in the coral tissue was found to be as high as $\sim$4.5% (*M. cavernosa*), $\sim$6.2% (red *L. hemprichii*) and $\sim$7.0% (orange *L. hemprichii*); these pigments thus constitute a considerable proportion of the soluble protein fraction.

Recently, statistical phylogenetic and mutagenesis analyses demonstrated that the evolution of the Faviina color diversity was driven by positive natural selection, and therefore the individual colors must have important functions [25,40]. Our analysis of the different tissues of *M. cavernosa* color morphs by multiple regression-based decomposition of fluorescence spectra supports this idea by revealing a distinct spatial distribution of different cyan, green and red pigments over different tissue types of the coral (supplementary Figs S4 and S5). Particularly striking is the close association of the long-wave green ($\sim$ 518 nm) fluorescent pigments [6] of *M. cavernosa* and the cyan FPs of *L. hemprichii* with the zooxanthellae (supplementary Fig. S4). The visual appearance of green and red colonies is correlated with a prevalence of cyan FPs (green morph) and red FPs (red morph) in the ectoderm of the coenosarc (supplementary Fig. S5). This observation is in good agreement with the differing levels of FP transcripts reported earlier [6], and underlines the importance of FPs for coral coloration. Further experimental work is required to determine the functions of the various pigment types.

Conclusions

In addition to pigments from zooxanthellae, green-to-red photoconvertible GFP-like proteins are the most important pigments responsible for the orange–red coloration of Faviina and Meandriina corals. Phycoerythrin was clearly excluded as a coloring compound in the above taxa. In contrast, zooxanthellae pigments show a light-dependent contribution to the visual appearance of *M. cavernosa*. The distribution of fluorescent proteins of different colors in coral tissues supports the hypothesis of specialized functions of the colors, although pinpointing them will require additional surveys and experiments. The absence of functional phycoerythrin in *M. cavernosa* shown in our study suggests that the observation of cyanobacterial symbionts in this species [16] needs to be reinvestigated.

Experimental procedures

Animal collection and storage

Corals were collected in Key West, FL, USA (*M. cavernosa*) (Permit No. FKNMS 2003-053-A1) or in the Great Barrier Reef, Australia (*L. hemprichii*) (Permit No. G01/268/2000). Additional specimens were purchased via the German aquaristic trade.

Preparation of tissue extracts

Tissue was removed from the corals with a scalpel and frozen at − 80 °C. Tissue was homogenized by sonication with a Branson sonic dismembrator (Branson, Danbury, CT, USA) (level 2/10%) in NaCl/P_i (50 mM sodium phosphate, pH 7.5, 150 mM NaCl) in an ice bath.

Determination of chlorophyll content

The zooxanthellae-containing pellets were resuspended in a saturated $MgCO_3$ solution. Algal pigments were extracted by adding acetone to a final concentration of 90%. Pigment concentrations were spectroscopically determined according to Jeffrey & Humphrey [32].

Construction of cDNA libraries and screening

Total RNA was extracted from 100 mg of fresh coral tissue following the protocol of Matz [42]. cDNA libraries were constructed using the SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA), and 5′/3′-RACE was performed, using the adapter primers in combination with degenerated primers. The sequence data were deposited at GenBank under the accession numbers EF186664 (cjarRFP) and EF186663 (lhemOFP).

Protein expression and purification, and determination of contents

Proteins were expressed and purified as previously described [20]. Photoconversion was achieved by irradiating the samples with a Sylvania 18 W fluorescent blacklight blue tube (Osram, Danvers, MA, USA) for 3 h at 4 °C.

Calibration series were set up using EosFP, mcavRFP and lhemOFP before and after photoconversion. Fluorescence intensity was measured at the green and red emission maxima, respectively. The amounts of protein in the calibration solutions and the cleared tissue extracts were determined following the manufacturer's instructions for the BCA protein assay (Pierce-Fisher Scientific, Pittsburgh, PA, USA). The content of fluorescent proteins was calculated from the red and green fluorescence of the tissue extracts in relation to the calibration series.

Fluorescence spectroscopy and lifetime analyses

The fluorescence spectra of the recombinant proteins were determined as previously described [20,33,34]. The relative contribution of individual FPs to the total fluorescence was determined by multiple regression decomposition of *in vivo* fluorescence spectra, using recombinant protein spectra as standards [6].

Immunochemical tests

The antigen solution was produced by mixing equal amounts of purified EosFP and mcavRFP. The antiserum (rabbit anti-EosFP/mcavRFP) was obtained after three immunizations of a rabbit with 50 μg of the antigen mix each.

Western blot analysis was performed according to standard procedures. The anti-EosFP/mcavRFP serum and diluted horseradish peroxidase-conjugated mouse anti-(rabbit IgG) F(c) were used at a dilution of 1 : 5000 (Rockland Immunochemicals, Gilbertsville, PA, USA), and visualized using a chemiluminescence system (ECL; Amersham Biosciences-GE Healthcare, Little Chalfont, UK). The same protocol was performed in parallel without adding antiserum in order to exclude a false-positive reaction of the secondary antibody. Double diffusion in gel analysis was set up in agarose gels (0.5%) in Petri dishes [35]. The antiserum was loaded in the central well and protein solutions in the surrounding wells. Plates were stored at 4 °C for at least 4 days to allow diffusion of the proteins. Fluorescence in gels was photographed using a hand-held blue light lamp (Nightsea, Andover, MA, USA) as excitation source, and a digital camera equipped with a red long-pass filter glass with a cut-off at 550 nm (Schott, Mainz, Germany).

Microscopic analysis

A detailed protocol of confocal imaging and spectroscopy of coral tissue is provided as supplementary material.

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Supplementary material

The following supplementary material is available online:

Doc. S1. Imaging of coral tissue.

Fig. S1. Multiple sequence alignment of green-to-red photoconverting proteins from *Dendronephtya* sp. (dendRFP), *Ricordea florida* (rfloRFP), *Scolymia cubensis* (scubRFP), *T. geoffroyi* (Kaede), *M. cavernosa* (mcavRFP), *C. jardinei* (cjarRFP), and *L. hemprichii* (lhemOFP, EosFP).

Fig. S2. Contribution of zooxanthellae pigments to the visual appearance of *M. cavernosa*.

Fig. S3. Western blot analysis of purified cjarRFP, lhemOFP, mcavRFP and EosFP.

Fig. S4. Confocal imaging of fluorescent pigments in red color morphs of *M. cavernosa* and *L. hemprichii*.

Fig. S5. Distribution of fluorescent proteins within the different tissues of the red and green color morphs of *M. cavernosa*.

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Supplementary Material

Supplementary Materials and Methods

Imaging of coral tissue. Polyp tissue of an orange color morph of *L. hemprichii* was dissected from an anesthetized animal. The anesthetizing solution was produced by mixing equal volumes of 7.5% $MgCl_2$ in distilled water and seawater. Samples were mounted on glass slides in seawater and sealed under a cover slip using Permount (Biomeda, Foster City, USA). Imaging of tissue fluorescence was done by single-photon confocal microscopy (Leica TCS/SP, Leica Microsystems Inc., Wetzlar, Germany) using a 20× objective. Fluorescence was excited with the 458 nm line of an argon ion laser and a RT 30/60 beam splitter. Green fluorescent proteins were imaged by their fluorescence at 490-520nm, orange-red fluorescent proteins were detected at 560-600 nm, and chlorophyll of zooxanthellae at 650-700 nm.

Colony fragments of different color morphs of the great star coral *M. cavernosa* (5-6 polyps) were fixed in 4% formaldehyde dissolved in 2× PBS (100 mM sodium phosphate, pH 7.4, 300 mM NaCl) for 48 h at 4ºC. After fixation, samples were rinsed in three changes of 2× PBS for 15-20 min. Samples were decalcified in neutral EDTA (10% EDTA, 1% NaOH, pH 7) for 72-96 h. The natural fluorescence of GFP-like proteins was not affected by the EDTA treatment. The decalcifying solution was replaced by fresh solution every 24 h. After decalcification, samples were rinsed three times for 15-20 min with 2× PBS and either immediately processed for histology or stored in 2× PBS at 4ºC for up to 4 weeks.

For sectioning, individual polyps were cut out from the decalcified coenosarc and incubated in 30% sucrose in PBS for 12-18h at 4ºC. Cryoprotected polyps were embedded in TissueTek (Sakura Finetech, Tokyo, Japan) and frozen at –80ºC for 5 min. Longitudinal sections 12-14 µm thick were cut at –22ºC on a Zeiss Mirome HM500 cryotome (Zeiss, Göttingen, Germany). Sections were thaw-mounted on gelatine-coated slides, briefly rinsed in 2x PBS to remove TissueTek and sealed with Permount under coverslips in 60% glycerol supplemented with the anti-fade agent p-phenylendiamine (PPD).

Whole sections were photographed with a Leica MZ FL III dissecting microscope (Leica) equipped with a mercury lamp, a set of filters for epifluorescence and a Spot Insight QE digital camera (Diagnostic Instruments, Sterling Heights, USA) while spectra of the tissue sections were collected using a USB2000 spectrometer (Ocean Optics, Dunedin, USA) equipped with a fiber optic probe that was coupled to the eyepiece of the microscope.

To document fine details of the distribution of fluorescent proteins in the tissues, stacks of optical sections (1-2 µm) were obtained on a Leica DM IRBE microscope equipped with a Leica SP2 LSCM confocal system and 488 nm Kr/Ar and 633 nm He/Ne lasers. Detection ranges were set to 500-550 nm for green fluorescent proteins, 560-650 nm for red fluorescent protein, and 660-720 nm for zooxanthellae.

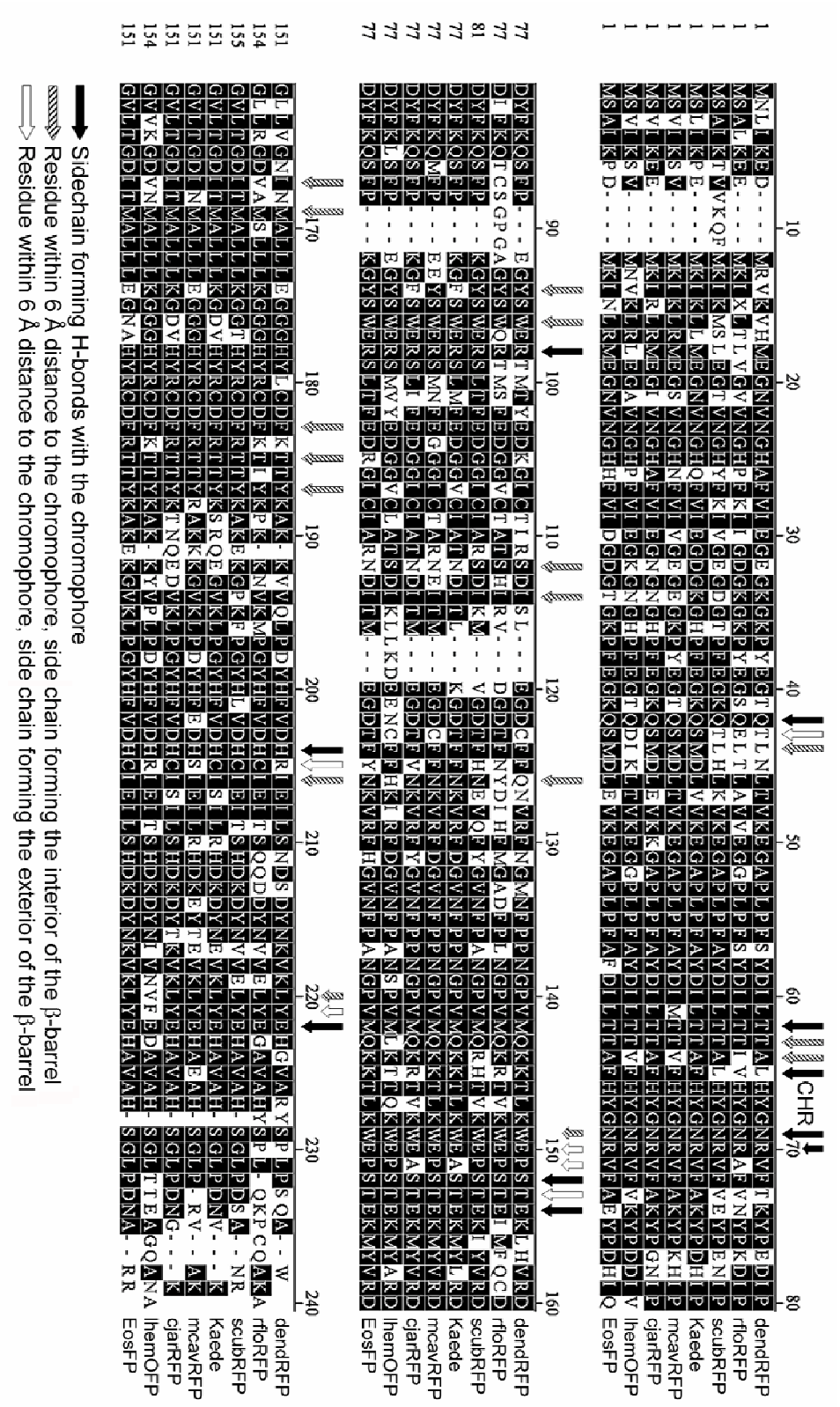

Figure S1: Multiple sequence alignment of green-to-red photoconverting proteins from *Dendronephtya sp.* (dendRFP), *Ricordea florida* (rfloRFP), *Scolymia cubensis* (scubRFP), *Trachyphyllia geoffroyi* (Kaede); *M. cavernosa* (mcavRFP), *Catalaphyllia jardinei* (cjarRFP), *L. hemprichii* (lhemOFP, EosFP). Residues identical among the majority of proteins are depicted with a black background. Amino acids localized within a 6 Å distance around the chromophore (CHR) are marked by arrows.

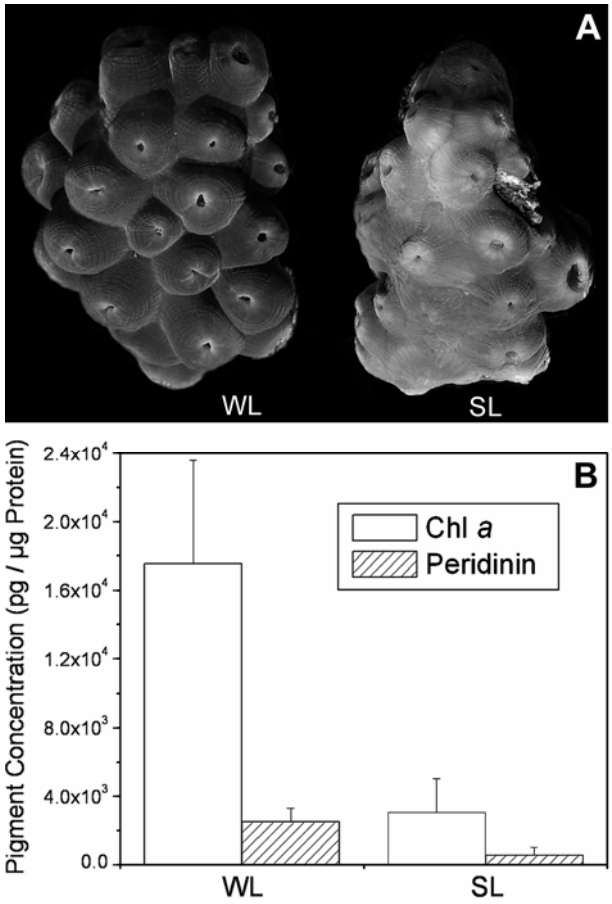

Figure S2: Contribution of zooxanthellae pigments to the visual appearance of *M. cavernosa*. (A) Replicates of one colony were kept under weak light (WL, 100 µmol m^{-2} s^{-1}) or strong light (SL, 400 µmol m^{-2} s^{-1}). The visually brighter appearance of strongly irradiated animals photographed under daylight is correlated with a reduced amount of the algal pigments chlorophyll *a* and peridinin (B).

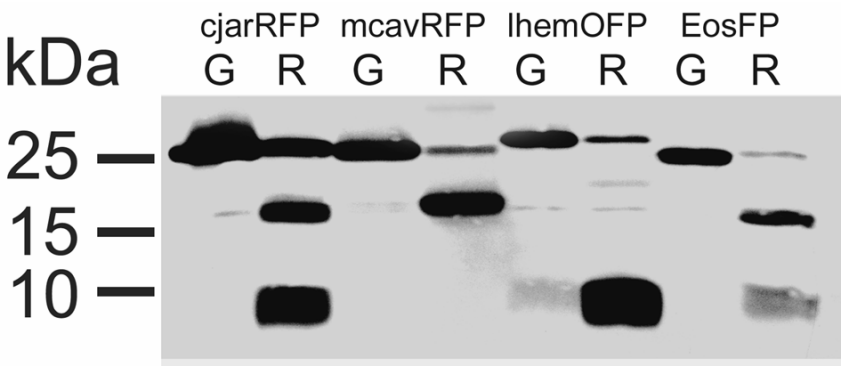

Figure S3: In the western blot analysis of purified cjarRFP, lhemOFP, mcavRFP and EosFP, the antiserum gives a strong signal for all unconverted proteins. After photoconversion, the antibodies bind to the ~20 kDa fragment of cjarRFP, mcavRFP and EosFP, but not of lhemOFP. The ~8 kDa fragment gets stained in the red form of cjarRFP, lhemOFP and, to a lesser extent, EosFP. In mcavRFP, there is no immunoreaction of the antiserum and the ~8 kDa fragment. The secondary antibody control produced only a negligible signal for similar exposure times (data not shown). G: Green form of the proteins; R: Red form.

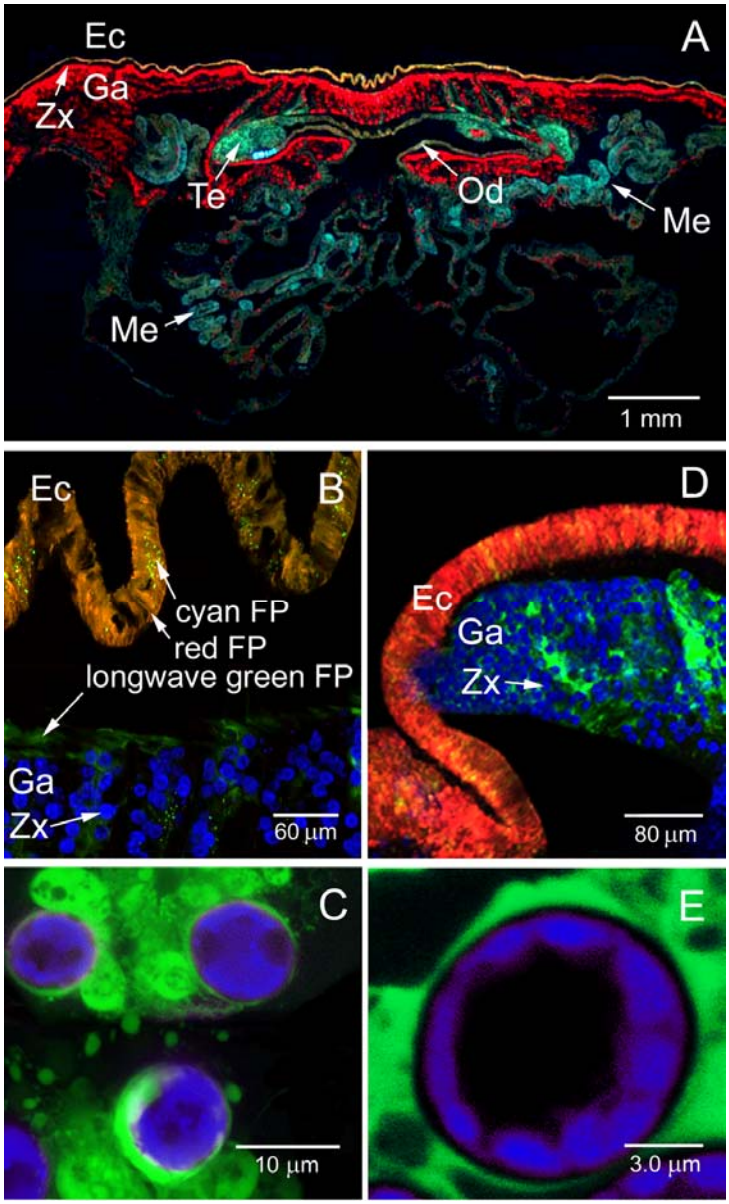

Figure S4: **Confocal imaging of fluorescent pigments in red color morphs of *M. cavernosa* and *L. hemprichii.*** A) Cross-section of a contracted polyp of *M. cavernosa* showing the distribution of cyan and red fluorescence in the ectoderm (Ec). Green fluorescence is dominant in the tentacle tissue (Te), the oral disk (Od) and mesenterial filaments (Me). Red chlorophyll fluorescence in the gastroderm (Ga) highlights the zooxanthellae (Zx). B) A closeup of the ecto- and gastroderm of *M. cavernosa* shows the localization of the different color types of FPs [6] as determined by spectroscopic analysis in the microscope. Zooxanthellae fluorescence was set blue. In some samples, also minor amounts of a red fluorescent pigment were detectable in the gastroderm (data not shown). C) Zooxanthellae can be found in close association with longwave green FPs in the gastrodermal tissue. D) *L. hemprichii* shows a similar distribution of fluorescent pigments between ecto- and gastroderm compared to *M. cavernosa*. E) A single zooxanthella cell from the gastroderm of *L. hemprichii* enclosed by GFPs.

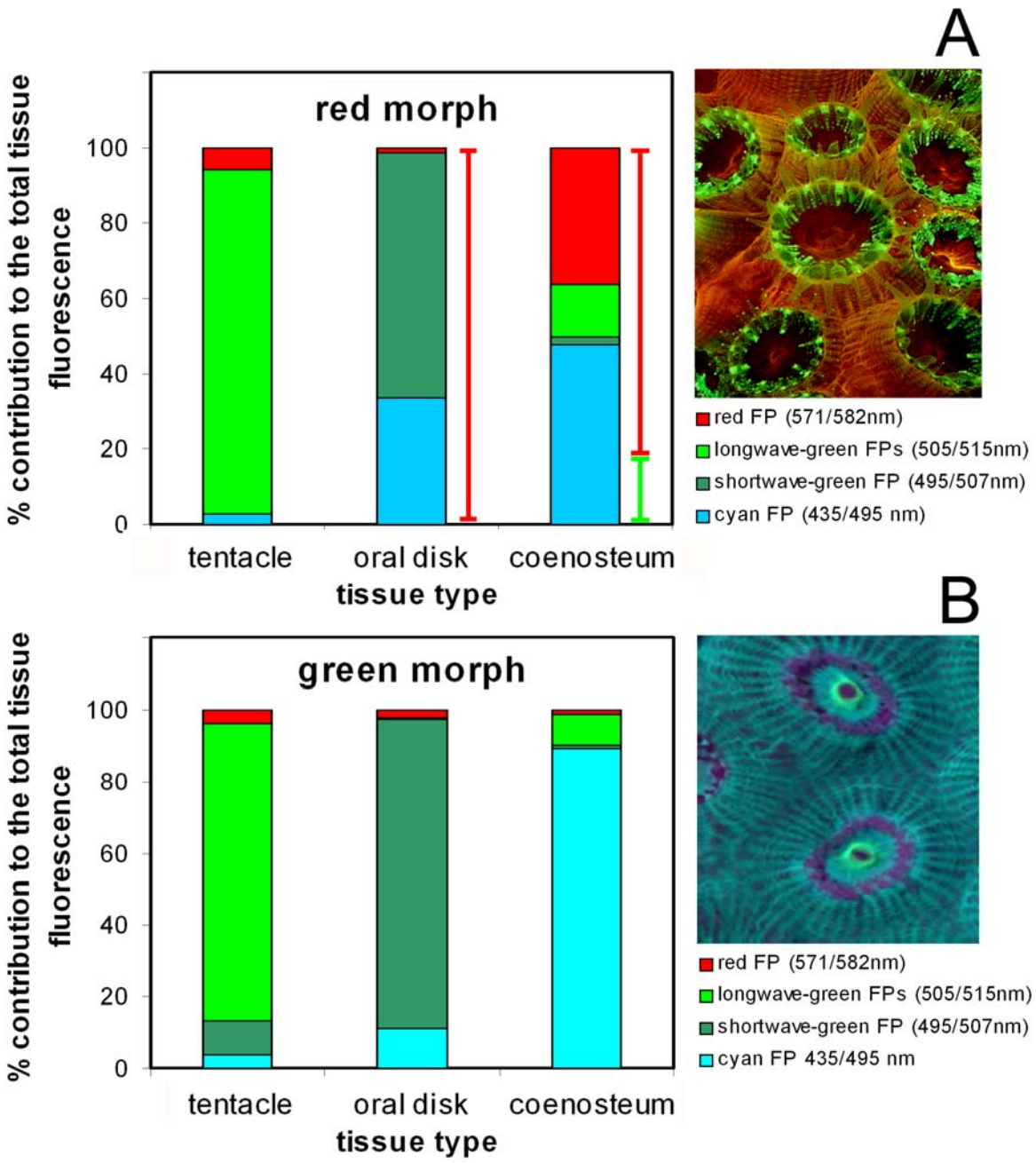

Figure S5: **Distribution of fluorescent proteins within the different tissues of the red and green color morphs of *M. cavernosa***. Contributions of the various FPs [6] were determined by multiple regression-based spectral decomposition. The color of the red morph is determined mainly by the coenosarc were major parts of the red FP are localized (A). The bars flanking the oral disk and coenosarc columns indicate the variability of pigments contents among different red colonies. The inset shows a fluorescence photograph of two polyps of the red morph. The cyan FP also localized in the coenosarc is responsible for the major coloration of the green morph (B). The inset shows the cyan and green fluorescence of two polyps of the green morph. The purple rings surrounding the mouths are due to reflected excitation light.