Original Article

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Dynamic Regulation of Fluorescent Proteins from a Single Species of Coral

Hung-Teh Kao,[1,2] Shelby Sturgis,[2] Rob DeSalle,[3] Julia Tsai,[4] Douglas Davis,[5] David F. Gruber,[6] Vincent A. Pieribone[5]

[1]Department of Psychiatry and Human Behavior, Division of Biology and Medicine, Warren Alpert Medical School, Brown University, Providence, RI 02912, USA
[2]Nathan Kline Institute and Department of Psychiatry, New York University School of Medicine, Orangeburg, NY 10962, USA
[3]Division of Invertebrate Zoology, American Museum of Natural History, New York, NY 10024, USA
[4]Skirball Institute Program of Molecular Neurobiology, New York University School of Medicine, New York, NY 10016, USA
[5]John B. Pierce Laboratory, Cellular and Molecular Physiology, Yale University, New Haven, CT 06519, USA
[6]Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ 08901, USA

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Abstract

To gain a better understanding of the natural function of fluorescent proteins, we have undertaken quantitative analyses of these proteins in a single species of coral, *Montastraea cavernosa*, residing around Turneffe atoll, on the Belizean Barrier Reef. We identified at least 10 members of a fluorescent protein family in this species, which consist of 4 distinct spectral classes. As much as a 10-fold change in the overall expression of fluorescent proteins was observed from specimen to specimen, suggesting that fluorescent proteins are dynamically regulated in response to environmental or physiological conditions. We found that the expression of some proteins was inversely correlated with depth, and that groups of proteins were coordinately expressed. There was no relationship between the expression of fluorescent proteins and the natural coloration of the *Montastraea cavernosa* specimens in this study. These findings have implications for current hypotheses regarding the properties and natural function of fluorescent proteins.

Keywords: barrier reef — Belize — coordinate expression — depth — *Montastraea cavernosa* — real-time quantitative PCR

Introduction

Scleractinian corals are one of the few Orders of Life to produce green fluorescent protein (GFP) homologs, and they do so in a variegated array of colors (Matz et al., 1999; Dove et al., 2000; Mazel et al., 2003). The function of these proteins remains unknown, but it has been hypothesized that they are photoprotective agents (Kawaguti 1944; Salih et al., 2000), antennas for photosynthesis (Schlichter and Fricke 1990), and possibly a nonenzymatic scavengers of radical oxygen species in the hyperoxic environment of corals (Mazel et al., 2003). At present, corals are becoming increasingly imperiled by bleaching events and climatic change (Pandolfi et al., 2003; Bellwood et al., 2004), increasing the urgency of understanding coral adaptability and resilience. Quantitation of fluorescent protein expression under different conditions would be highly informative, as it may elucidate the role and function of fluorescent proteins in relation to coral survival. It is not known if the expression of these proteins in healthy corals is constant, or if the expression is dependent on environmental conditions. There have been few such studies on this topic owing partly to the challenges associated with quantifying expression of fluorescent proteins in coral. Corals possess a tissue thickness of only two cell layers, epidermis

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Correspondence to: Hung-Teh Kao; E-mail: Hung-Teh_Kao@brown.edu
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and gastrodermis, covering the calcium carbonate skeleton (Sorokin 1993), limiting the amount of tissue obtainable under field conditions.

Several different sequences of fluorescent proteins have been cloned from a single coral species, leading to the suggestion that these proteins are derived from a multigene family that may have arisen through gene duplication (Kelmanson and Matz 2003). Molecular evolutionary studies also suggest that the ancestral fluorescent protein was probably green, and that red fluorescent proteins evolved through small incremental transitions from the ancestor (Ugalde et al., 2004). These studies emphasize that fluorescent proteins have flourished and evolved during the existence of coral, which first appeared in the Upper Triassic period (Stanley and Swart 1995), suggesting that fluorescent proteins have ancient origins, but they do not provide any clues as to their function.

In this study, we quantitated the expression of fluorescent proteins from *Montastraea cavernosa*, one of the most abundant coral species in the Caribbean. Several fluorescent proteins have already been cloned and characterized from *Montastraea cavernosa* (Kelmanson and Matz 2003; Carter et al., 2004; Sun et al., 2004), making this organism an ideal candidate to explore the biology of fluorescent proteins in their natural habitat.

Materials and Methods

Collection and Documentation of Specimens. Specimens were collected under a permit from the Government of Belize. Specimens of *Montastraea cavernosa* were collected on the Turneffe atoll over a period of 2 days. Specimens were selected from exposed reefs, avoiding areas that lacked exposure to sunlight such as under overhangs or caves. Depth was recorded for each sample at the time of collection. Images of the specimen were collected underwater in situ via a Nikon Coolpix 2000 camera in an Ikelite case and an Ikelite DS1000 digital strobe. Samples were photographed using identical focus, aperture, exposure time, flash intensity and duration, and focal distances. White balance was precalibrated on land and fixed for all exposures. Image color and exposure parameters were not manipulated before representation in this communication.

Identification of *Montastraea cavernosa* was made by visual identification (Figure 1). In several instances, we were able to confirm species identity by direct sequencing of a fragment of the mitochondrial cytochrome oxidase gene (*MCO*), which was amplified from either the cDNA or genomic DNA. *MCO* primers and conditions for amplifying this sequence have been previously described (Fukami et al., 2004).

RNA Extraction. Small fragments of each colony were collected and rapidly processed for extraction of total RNA. RNA extraction was conducted in a clean area using precautions to prevent RNase contamination. Coral samples were rinsed in Instant Ocean (Aquarium Systems, Mentor, OH) supplemented with 10 U/ml of RNAguard (Amersham Biosciences, Piscataway, NJ). Sharp instruments were used to excise soft coral tissue from the calcium carbonate skeleton. RNA was extracted from this tissue via RNeasy columns (Qiagen, Valencia, CA).

cDNA Synthesis and Cloning of Fluorescent Proteins. cDNA synthesis was initiated by oligo-dT or random hexamers using Stratascript reverse transcriptase (Stratagene, La Jolla, CA). To clone fluorescent proteins from *Montastraea cavernosa*, we designed a conserved 5′ primer corresponding to a region of the mRNA that included the start codon (CTTA CTTACGTCTACCATCATGAGTGTG), and a 3′ primer that included the stop codon (TTG GCTTTTCGTTAAGCCTTTACTTGGCC). Both primers were directed against regions that were conserved among fluorescent proteins previously cloned from *Montastraea cavernosa* (Kelmanson and Matz 2003; Carter et al., 2004; Sun et al., 2004). These two primers were used to amplify the entire coding region of the cDNA corresponding to fluorescent proteins, and polymerase chain reaction (PCR) products were cloned into the vector pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA). Fluorescent proteins were constitutive expressed, because they were cloned in-frame downstream of the lac promoter. Expression was visualized by plating bacteria onto CircleGrow agar plates (MP Biomedicals, Irvine, CA) supplemented with kanamycin (20 μg/ml) and charcoal (2% wt/vol) to suppress endogenous fluorescence from bacterial media. Colonies were visualized using Illumatool (Lightools Research, Encinitas, CA).

Emission Spectra. Emission spectra were determined using an Ocean Optics USB2000 spectrophotometer with a hand-held fiber optic probe. Excitation was achieved by specific band pass filters, kindly provided by Chroma Optics, Inc., VT. The emission spectrum of individual, cloned fluorescent proteins was achieved by applying the fiber optic probe to single bacterial colonies expressing the protein.

Molecular Phylogeny. Sequences homologous to fluorescent proteins cloned from Belize were

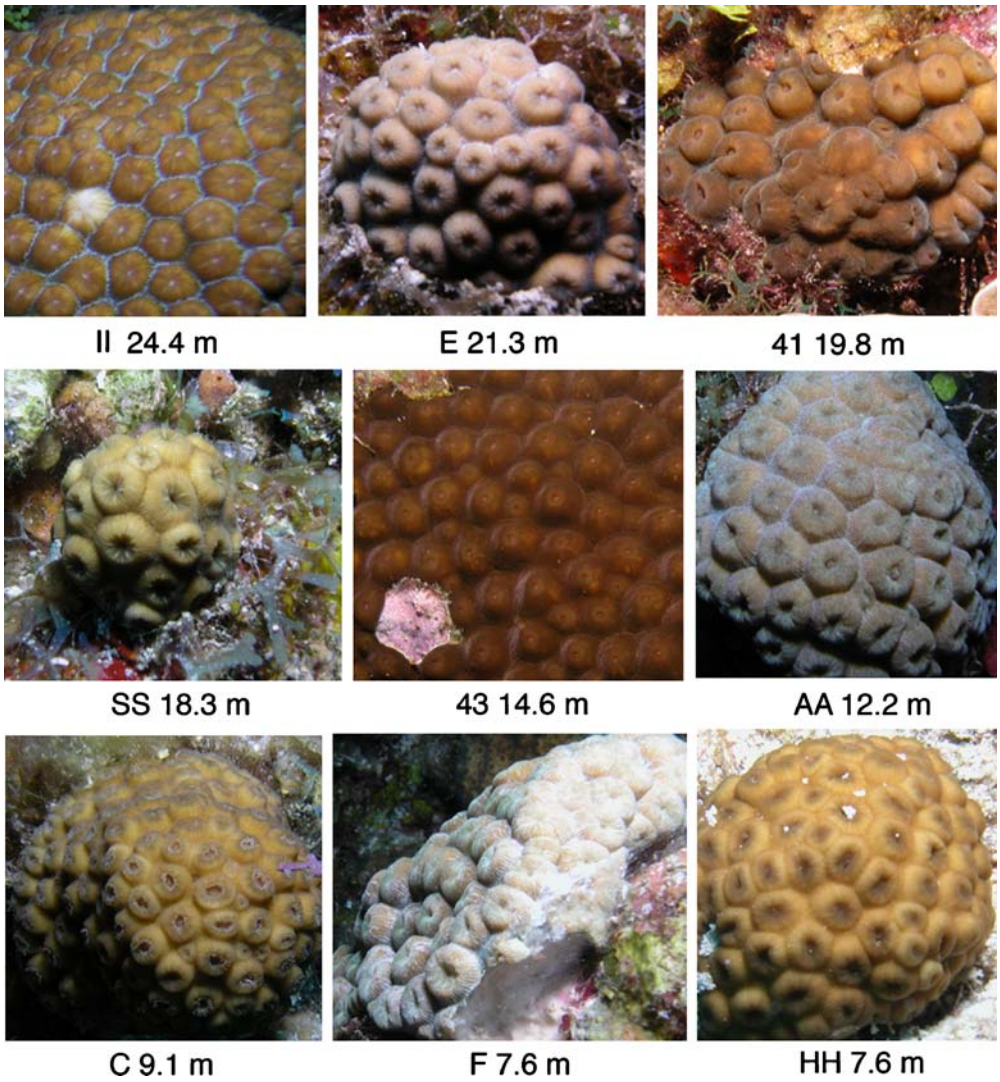

Figure 1. Underwater photographs of some of the coral specimens used in this quantitative study. The specimen name and depth correspond to those used in Table 4.

retrieved from GenBank using the BLASTp program. More than 100 protein sequences from thirty organisms were obtained (Supplementary Table 1). A matrix was generated in NEXUS file using Clustal (Higgins and Sharp 1988) with default alignment parameters in effect. The alignment generated was generally unambiguous with few hypothesized indel events. The NEXUS file was then analyzed in PAUP (Swofford, 2002) using 100 replicates of random taxon addition and tree bisection reconnection (TBR) branch swapping. This approach ensures that the tree space is searched adequately. To assess the robustness of the phylogenetic inference we performed both bootstrap and jackknife analyses in PAUP using 10,000 replicates of both resampling techniques. In addition, Bayesian posterior probabilities were generated using MrBayes (Huelsenbeck and Ronquist, 2001), using MCMC with 1,000,000 simulations with burnin as default settings and with parsmodel in effect. These results are reported in Figure 2, where to make the figure more concise, we have grouped the results into four different classes: 100/100/1.00 (bootstrap/jackknife/Bayes probability); 80–90%/80–90%/0.95–1.0; <80%/<80%/0.95–0.9; and <NS/NS/0.8–0.9. In addition, we used MacClade (Maddison and Maddison, 2000) to map the emission wavelength for the various fluorescent proteins on the tree. We used Af_gfplike as an outgroup, as it was placed consistently outside of the larger group of GFP homologs we focused on in this study.

Real-Time RT-PCR. RNA samples were digested with 50 U/100 μl of RNase-free DNase (Invitrogen) for 30 min at 37°C, and then repurified using an

Table 1. Primer sets used to amplify specific sequences

Primer set	*Primers*	*Sequence*	*Final concentration (nM)*
ALL1	ALL1-5′A	ATACCAGACTATTTCAAGCAGWCGTTTCC	375
	ALL1-5′B	ATACCAGACTATTTCAAGCAGATGTTTCC	375
	ALL1-5′C	ATACCAGATTATTTCAAGCAGACGTTTCC	375
	ALL1-3′A	TCTTCYTCTGCATAACTGGACC	1500
ALL2	ALL3-5′B	GAAGGAGGTGGCCATTACC	1700
	ALL3-3′B	AGAATGAGCTTCGGCATGC	1700
ALL3	ALL3-5′A	GAAGATCAAGCTGCGTATGG	3200
	ALL3-3′A	GTCAAGATATCGTAAGCGAAAGG	2800
1a	Gp1-5′B	AGGGCAAAGAAAGGTGTCG	1300
	Gp1-3′B	CTTAATCGGCAGCATAGAAGG	1200
1b	Gp1 F	GATGTTAGCAGGACGCTGTTG	600
	Gp1 R	AATTTCAATTCGGTGGTCCACAAAG	500
1 and 2	Gp1/2 3′C	GTAGCACTCCATCACGCACG	300
	Gp1/2 5′C	GACTTTCGAAGACCAGGGCG	650
3a	Gp3/7a-F	CTGGGAACGAAGCATGACATA	600
	Gp3/7a-R	CGAATTTTATAGACAAAACAGTCGTCGA	500
3b	Gp3/7a-F	CTGGGAACGAAGCATGACATA	600
	Gp3/7b-R	GCATAACTGGACCATTGGCA	650
4	Gp4-5′	AGCATGACTTTCGAAGACGG	450
	Gp4-3′	GAAAGTTCACACCATCAAATCGAAC	350
5	Gp5-5′	ATGAAGACCAGAGTATTTGCACG	400
	Gp5-3′	CATCACGCACATACATTTTCTCG	400
10	Gp10-F2	GGGCACAACTTCACGATTGTT	1200
	Gp10-R1	CAGAGGTCCGCCTTCTAC	1500
18S	18S-F2	TGCTGGGGATAGATCATTGC	3200
	18S-R2	TCAGAAGGCCTCACTAAACC	3300
β-tub	mcB-tubF	CTCACCACACCAACTTATGG	300
	mcB-tubR	AAGGAACCATGTTCACTGCC	300

RNeasy column before reverse transcriptase PCR (RT-PCR).

Typical reaction conditions (20 μl) were: 1× Stratascript buffer, 50 to 250 ng of total RNA, 1 mM deoxynucleotide triphosphates, 7.5 ng/μl of hexamers, 20 U of RNAguard, and 40 U of Stratascript reverse transcriptase. The reactions were incubated at 25°C for 10 min, then 42°C for 60 min, and finally 72°C for 10 min. Two units of RNase H were then added and the reactions were incubated for another 30 min at 37°C. The reactions were then diluted with 85 μl of water, and 6.25 μl was used in each PCR. Controls lacking reverse transcriptase consisted of the same composition, except water was substituted for reverse transcriptase.

Real-time PCRs were performed in 96-well plates using the ABI7700HT DNA Detection System (Applied Biosystems, Haywood, CA). All reaction volumes were 25 μl. To each reaction was added 6.25 μl of a 4× mix of primers (Table 1), 6.25 μl of diluted RT template, and 12.5 μl of 2× Full Velocity Mix (Stratagene). The cycling conditions were identical for each set of primers used. After a heat activating step of 95°C for 10 min, the reactions were cycled through 95°C for 10 s followed by 60°C for 20 s for a total of 40 times. Data were acquired throughout the cycling in the presence of the passive dye indicator, ROX. C_t values were obtained via SDS1.1 software (Applied Biosystems).

RNA standards were analyzed with every PCR run. The standards consisted of a serially diluted combination of RNAs corresponding to each fluorescent protein cDNA, β-tubulin, and 18S rRNA spanning four orders of magnitude. These RNAs were transcribed in vitro from plasmids using an RNA transcription kit (Ambion, Austin, TX), and individual purified RNAs were quantitated via the Ribogreen assay (Invitrogen). RNA standards were

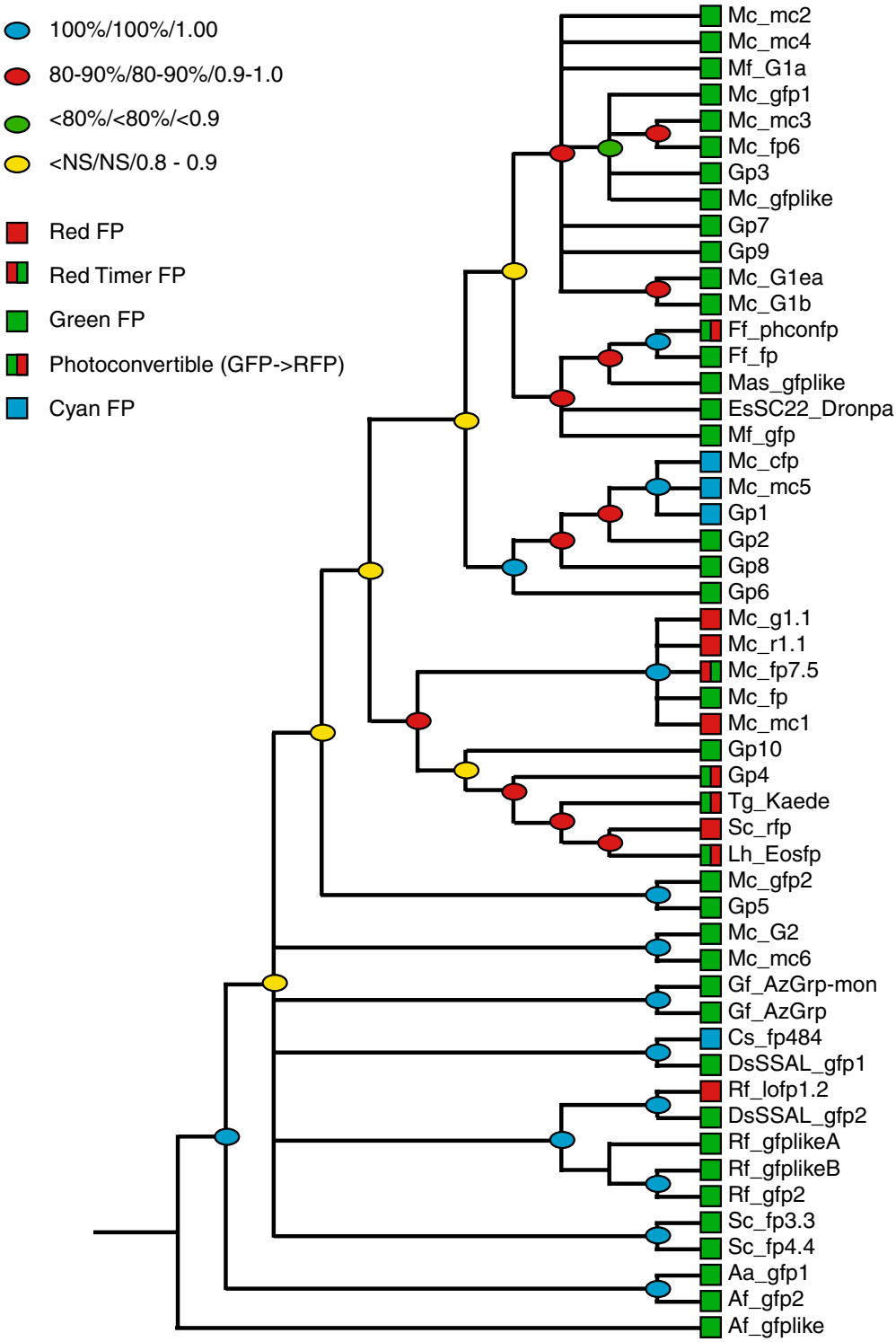

Figure 2. Molecular phylogeny of the cloned fluorescent proteins, compared to other known fluorescent proteins. About 40 other fluorescent proteins were clustered with those cloned from *Montastraea cavernosa* from Belize. The protein names used in this tree are the same as those used in the GenBank submission, and are listed in Supplementary Table 1. Sequences were aligned and analyzed in PAUP as described in Materials and Methods. The tree depicted represents a strict consensus of more than 200,000 equally parsimonious trees. Nodes have been color-coded into four different classes as indicated: 100/100/1.00 (bootstrap/jackknife/Bayes probability); 80–90%/80–90%/0.95–1.0; <80%/<80%/0.95–0.9; and <NS/NS/0.8–0.9. The emission color of the fluorescent proteins has also been color-coded as indicated.

subjected to the same RT-PCR reaction as the coral RNAs. A standard curve generated by the RNA standard, which converts C_t to fmol, allowed us to determine the absolute amount of initial RNA in the original coral sample (User Bulletin 2 2001).

Real-time PCR was performed at least three times using the same set of primers to ensure reproducibility. The absolute amount of specific RNA in each sample was also adjusted by subtracting the calculated amount obtained in absence of reverse transcriptase. Because each sample had varying levels of total RNA, the relative level of RNA expression was determined by normalizing the absolute amount to 18S rRNA. 18S rRNA amplification was carried using primers directed against a region that is highly conserved among different coral species, including *Montastraea cavernosa* (Berntson et al., 1999). We documented the level of expression for a specific gene as the logarithm of (fmol of gene RNA $\times$ 10^6)/(fmol of 18S rRNA). We used this logarithm because it is proportional to the value that is actually measured by real-time quantitative PCR, the C_t value. A "housekeeping", structural gene, β-tubulin, was also used as an additional control. Again, we employed primers directed against a conserved region of this molecule to quantitate the mRNA (Fukami et al., 2004).

Software. DNA sequence analyses was performed using DNAStar (Madison, WI). This program was also used to design and evaluate primers for real-time PCR. Statistical analyses were performed using Statview (SAS, Carey, NC) or Microsoft Excel. Graphs were drawn in Igor Pro 5 (Wavemetrics, Portland, OR).

Results

Multiple Fluorescent Proteins Are Expressed in Montastraea cavernosa. Initially, amplified cDNA libraries from nine specimens of *Montastraea cavernosa* were analyzed. All specimens were visually identified as members of this species (Figure 1). In 4/9 cases, we were able to confirm species identification by sequencing a fragment of the mitochondrial oxidase gene, amplified from either the cDNA or genomic DNA. In 4/9 cases, the PCR reactions failed to provide sequence data. In 1/9 case, the *MCO* amplified was that of *Mussa angularis*, which differs radically from *Montastraea* morphologically, and is a spurious result most likely explained by cross-hybridization between two species of coral, RNA editing, or larval contamination.

Approximately 100 different cDNA clones were sequenced from the 9 specimens of *Montastraea cavernosa*, and upon alignment, we found a minimum of 10 different fluorescent proteins (termed Gp1 to Gp10) potentially expressed from this single species of coral. Although some of these proteins were cloned only once, several were cloned multiple times, with Gps 1, 2, 3, 4, and 7 represented multiple times in this initial screen. All fluorescent proteins

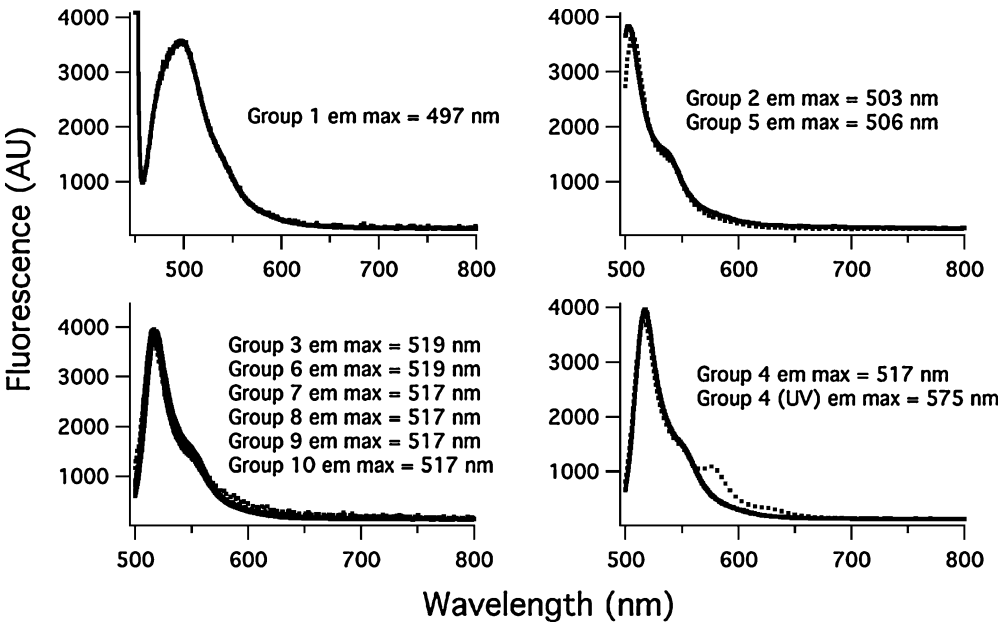

Figure 3. Emission spectra for the 10 fluorescent proteins identified from *Montastraea cavernosa*. Fluorescent proteins were individually expressed in *E. coli* on charcoal agar plates, and emission spectra were obtained using an Ocean Optics USB2000 spectrophotometer with a hand-held fiber optic probe.

are homologous, but not identical, to fluorescent proteins previously identified from *Montastraea cavernosa* (Kelmanson and Matz 2003; Carter et al., 2004; Sun et al., 2004).

Each cloned fluorescent protein was expressed in *E. coli* and their spectral characteristics examined. The emission spectrum for each of the 10 fluorescent proteins is shown in Figure 3. We found that a range of fluorescent colors was represented by this set of cDNAs: Gp1 was a cyan fluorescent protein (emission maxima = 497 nm); Gps 2 and 5 were predominantly blue-green (emission maxima = 503 to 506 nm); and the remainder green (emission maxima = 517 to 519 nm). One protein, Gp4, was a Kaede-like, photoconvertible protein (Ando et al., 2002) that switches from a green fluorescence to a red fluorescence on ultraviolet (UV) irradiation. The proteins also displayed varying degrees of brightness upon expression in *E. coli*, with Gp10 the dimmest, and Gps 2 to 7 the brightest to the unaided eye. Thus, *Montastraea cavernosa* expresses a range of fluorescent proteins with varying spectral properties.

Molecular Phylogeny of the Fluorescent Proteins. We retrieved more than 100 fluorescent protein sequences that were related to Gps 1 to 10 from GenBank (Supplementary Table 1). Because the Gps 1 to 10 protein sequences were clustered among about 40 other proteins, we restricted our analysis to these proteins. In general, we found that the cDNAs cloned from Belize were sequestered to clades containing other fluorescent proteins derived from *Montastraea cavernosa* (Figure 2). This latter result indicates that all the cDNAs obtained from these specimens are most likely from a single species, *Montastraea cavernosa*, and that the single *Mussa MCO* taxonomy identification is an anomaly. The parsimony analysis resulted in more than 200,000 equally parsimonious trees for which a strict consensus tree was constructed (Figure 2). Robustness of nodes for this parsimony tree was generally strong at the tips of the tree with some erosion near the base. The major groups of fluorescent proteins in corals generally coincided with the color of their emission, and this was strongly supported by the various analyses. In particular, the green proteins were supported with high Bayes probability (0.99) and relatively good bootstrap values (67%), the cyan proteins were strongly supported by all three approaches (100%/100%/1.00), and the red proteins were also supported as a group at high Bayes probability (0.99) and reasonable bootstrap and jackknife values (65% to 70%). The base of the tree, which contains proteins from several other species, shows consistent strong support using all three approaches.

The proteins isolated in this study grouped into four areas of the tree (Figure 2). Gps 3, 7, and 9 grouped with existing GFPs, including the *Montastraea cavernosa* proteins mc2, mc3, and mc4 (Kelmanson and Matz 2003), which have similar emission wavelengths (515 ± 3 nm). Gps 1, 2, 6, and 8 grouped with cyan fluorescent proteins, including mc5 that was previously described with an emission peak of 495 nm (Kelmanson and Matz 2003). Gps 1 and 2 have emission peaks consistent with cyan fluorescent proteins (497 and 503 nm, respectively), but Gps 6 and 8 have emissions similar to other GFPs. Gps 4 and 10 group with red fluorescent proteins, including mc5, which has a peak emission at 585 nm (Kelmanson and Matz 2003), as well as Kaede (Ando et al., 2002) and another Kaede-like protein, EosFP (Wiedenmann et al., 2004). The emission spectrum of Gp4 is consistent with this grouping, while Gp10 resembles other GFPs. Finally, Gp5 falls outside of the three previous classes, but is located in the same part of the tree that includes mc6, which has a similar emission peak (507 nm) (Kelmanson and Matz 2003).

Validation of the Real-Time PCR Quantification Method. We were interested in quantitating gene expression of individual fluorescent proteins, and given the limited quantities of tissue available, real-time quantitative PCR appeared to be the best method for this type of analyses. The cDNAs encoding each of the different fluorescent proteins displayed pairwise similarities ranging from 81% to 96.7% (using DNAStar), suggesting that primers unique to a given cDNA might be challenging to find and verify. We sought primer pairs that were capable of amplifying individual cDNAs as well as the entire set of fluorescent protein cDNAs. The selection of primer sequence was based on inspection of the aligned sequence, and directed toward regions that were relatively specific for each fluorescent protein. We tested 47 different primer combinations at varying concentrations, of which 13 were eventually used on coral samples (Table 1). The primer sets were extensively characterized by performing pilot real-time PCR tests on a panel of diluted cDNAs (Table 1).These results were used to establish the specificity of the pairs. The amount of template used in each reaction (0.6 fmol) greatly exceeds the amount expected in a coral sample, thus providing a rigorous test as to whether the primers recognize nonspecific sequences under the conditions used. Some primer sets were capable of selectively amplifying a single fluorescent protein cDNA (e.g., 5 and 10), but other primer sets amplify more than one cDNA (e.g., 3b).As an additional measure to validate the real time RT-PCR method, we were able, in some cases, to design

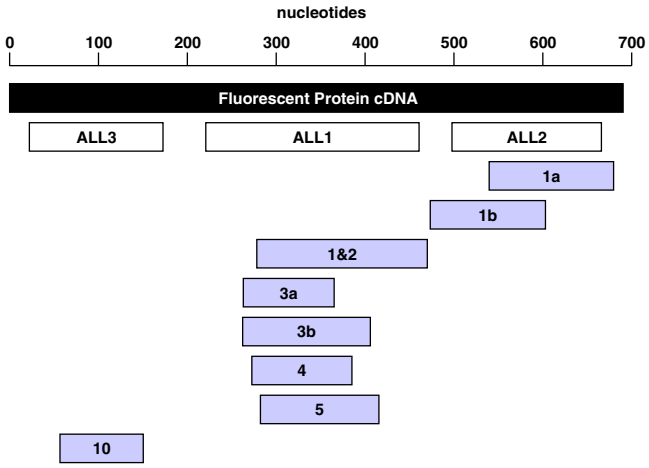

Figure 4. Schematic diagram illustrating the regions of DNA amplified during real-time PCR. The region of DNA amplified by specific primer sets (Tables 2 and 3) is mapped to a location on the full-length cDNA. Note that some primer sets that are capable of recognizing the same complement of proteins are directed toward different regions of the cDNA (e.g., ALL1 and ALL2, 1a and 1b).

more than one set of primers that recognize the same transcript (Figure 4). All the primer sets designed to amplify fluorescent protein sequences failed to amplify unrelated *Montastraea cavernosa* sequences (18S rRNA, β-tubulin, MCO, or ran1) or vector sequence (Table 2).

In our initial tests, we used a PCR master mix from Applied Biosystems, a standard reagent in real-time PCR. Although this mix performed well in pilot tests involving reversed transcribed synthetic RNA, no signal could be obtained from cDNA derived from coral RNAs. Troubleshooting revealed an incompatible component in this PCR master mix. Another PCR master mix, the Full Velocity Mix (Stratagene), performed well on both the coral and pilot tests, and was used in all subsequent analyses.

Comparison of β-Tubulin and Fluorescent Protein Expression Levels. We first compared the expression of β-tubulin, a structural gene, to 18S rRNA (Table 3). The values obtained from these measurements did not vary greatly from sample to sample (standard deviation = 0.18), suggesting that β-tubulin trans cription was relatively stable. However, when β-tubulin expression plotted was against water depth of the specimen, we found a surprising positive correlation: as depth increased, so did β-tubulin expression (Figure 5).

By contrast, the expression of fluorescent protein transcripts found in each coral sample varied considerably (standard deviation = 0.37–0.50) (Table 3). For example, the highest relative expression of fluorescent proteins was observed in sample F, equaling or exceeding that of β-tubulin, while the relative expression in sample C was 10% that of β-tubulin. Unexpectedly, these differences were not related to the color or apparent fluorescence intensity of the specimen in an obvious way. The specimens that expressed the highest levels of Gp1 RNA, which encodes a CFP-like protein, were E and C. The specimens that expressed the highest levels of Gp4 RNA, encoding a Kaede-like GFP to RFP converting protein, were II and F. Of the transcripts we were able to quantitate, Gp10 was of the lowest abundance. This protein was also the dimmest and took longer to mature than any of the other proteins.

Correlation of Fluorescent Protein Expression with Depth. Using three different primer sets to measure the overall quantity of fluorescent proteins, we found that expression of all fluorescent proteins as a group did not vary with depth (up to 24.4 m) (Figure 5). No relationship with depth was found using relative quantities normalized to 18S rRNA, or when these values were normalized to the levels of β-tubulin.

We next examined the relative levels of expression for individual fluorescent proteins with depth (Figure 6). While the number of samples was small, we observed an inverse correlation of RNAs for Gp5 and possibly 10 with depth. By contrast, we observed no correlation with depth for RNAs encoding Gps1, 3, 4, or 3 and 7 combined.

Coordinated Expression of Fluorescent Protein Expression. Because the expression of some individual RNAs correlated with depth, but others did not, we investigated whether there was a pattern of expression for groups of RNA. Pairwise correlations were performed on expression levels in the various specimens (Table 4) to determine whether there was coordinate expression of some RNAs. We observed positive correlations between quantities obtained using primers sets 1a with 5, 1a or 1b with 10, 1 and 2 with 10, and 5 with 10 (Table 4).These findings suggest that the individual RNAs for Gps 1, 5, and 10, are expressed similarly, and could be under the control of a common regulator.

We also observed a positive correlation between the expression of Gp1 (primers sets 1a and 1b) and the combination of Gp1 and 2 (primer set 1 and 2), suggesting that Gp2 RNA may be coordinately expressed along with Gp1. Similarly, there was a positive correlation between the expression of Gp3 (primers set 3a) and the combination of Gp3 and 7 (primer set 3b), suggesting that Gp7 RNA may be coordinately expressed along with Gp3.

Table 2. Specificity of primers for the detection of cDNA

	Templates														
Primer set	*Gp1*	*Gp2*	*Gp3*	*Gp4*	*Gp5*	*Gp6*	*Gp7*	*Gp8*	*Gp9*	*Gp10*	*β-tub*	*18SA*	*18SB*	*ran1*	*MCO*	*Vector*
ALL1	***	***	***	***	***	*	***	***	***	**	0	0	0	0	0	0
ALL2	0	***	***	**	***	*	***	***	***	***	0	0	0	0	0	0
ALL3	**	**	**	**	**	*	**	**	**	**	0	0	0	0	0	0
1a	***	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1b	**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 and 2	***	***	**	0	0	*	*	***	**	0	0	0	0	0	0	0
3a	0	0	**	0	0	0	0	0	0	0	0	0	0	0	0	0
3b	0	0	***	0	0	0	***	0	0	0	0	0	0	0	0	0
4	0	0	0	***	0	0	0	0	0	**	0	0	0	0	0	0
5	0	0	*	0	***	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	***	0	0	0	0	0	0
18S	0	0	0	0	0	0	0	0	0	0	0	***	0	0	0	0
β-tub	0	0	0	0	0	0	0	0	0	0	***	0	0	0	0	0

Real-time PCRs were carried out on templates (indicated horizontally) using primers sets (indicated vertically) described in Table 1. The resulting C_t value was tabulated using asterisks to designate the degree of sensitivity as follows: *** for C_t values from 10 to 15; ** for C_t values from 15 to 20; * for C_t values from 20 to 25; and 0 for C_t values >25. All templates are cDNAs cloned into the vector pCR4Blunt-TOPO.The templates 18SA and 18SB refer to the 5′ half and 3′ half of the cDNA. The clone ran1 is a random cDNA obtained from *Montastraea cavernosa*. Vector DNA consists of pCR4Blunt-TOPO with no insert.

Correlation of Fluorescent Protein Expression with Coloration of Coral. We examined a wide range of color morphs of *Montastraea cavernosa*. Underwater these corals often appeared red or green to the unaided human eye. However, in flash photographs, their colors were mostly muted greens and browns. Because blue dominates the color spectrum at deeper depths, this could reduce the contrast of red and green fluorescence, giving rise to the muted colors. We found that a color correction filter that reduced the level of blue light at depth significantly restored this contrast, so that the colors observed in photographs corresponded to underwater observations (Figure 1).

When corals were brought to the surface, their fluorescent color disappeared in sunlight. They were then visualized in the dark, using restricted bandpass illumination. Under these conditions, the emission spectrum collected from the surface of the coral was dominated by one of four peaks (497, 506, 517, and 575 nm), and occasionally, minor combinations of other peaks (data not shown). These peaks corresponded to those of the recombinant fluorescent proteins cloned from these specimens (Figure 3), and were similar to previous emission spectra of fluorescent proteins found in *Montastraea cavernosa* (Kelmanson and Matz, 2003).In addition to emission in the 497 to 575 nm range, we observed emission at 680 and 736 nm, which corresponded to chlorophyll fluorescence. We observed no consistent correlation between fluorescence color and the natural coloration of color. We also observed no correlation between the color of the coral and the expression levels of various proteins (Figure 7), or any correlation of natural (Figure 1) or fluorescent color with depth.

Discussion

We examined the types and quantities of fluorescent proteins produced in samples of *Montastraea cavernosa* collected from Turneffe atoll on the Belizean Barrier Reef. This species expresses at least 10 different fluorescent proteins (Gps 1 to 10), which is consistent with previous reports indicating that many fluorescent proteins can be cloned from this organism (Kelmanson and Matz, 2003).The proteins exhibit four different fluorescence emission spectra, likely arising from two different chromosphere structures (Kelmanson and Matz, 2003).

Molecular phylogenic analyses segregated the cloned proteins roughly into clades corresponding to their emission spectra, and hence to their color.

Table 3. Relative expression of β-tubulin and all fluorescent proteins in *Montastraea cavernosa*

Primers:	*β-tub*		*ALL1*		*ALL2 and 1a*		*ALL3*		*Depth*
SAMPLE	*Log*	*SEM*	*Log*	*SEM*	*Log*	*SEM*	*Log*	*SEM*	*(m)*
II	2.91	0.18	1.82	0.22	2.49	0.28	1.19	0.11	24.4
XY	2.70	0.11	1.25	0.42	2.91	0.14	1.23	0.17	24.4
E	2.79	0.21	2.21	0.19	2.50	0.38	1.70	0.03	21.3
SS	2.82	0.11	2.02	0.17	1.79	0.03	1.99	0.23	18.3
AA[b]	2.65						1.96		12.2
C	2.49	0.29	1.32	0.52	1.56	0.16	1.40	0.03	9.1
F	2.38	0.13	2.81	0.28	2.40	0.21	2.32	0.13	7.6
HH	2.62	0.31	1.75	0.21	1.56	0.12	1.55	0.26	7.6
4	3.38[a]	0.20	2.46	0.13	2.08	0.09	2.11	0.15	13.7
11	2.54	0.29	2.09	0.34	2.22	0.18	1.32	0.02	13.7
41	2.94	0.09	2.20	0.10	1.25	0.05	1.58	0.13	19.8
43	2.79	0.12	2.42	0.08	2.30	0.28	1.77	0.31	14.6
SD	0.18		0.47		0.50		0.37		

Real-time PCRs were carried out for each sample as described in Materials and Methods. Absolute quantities obtained using each primer set were normalized to 18S rRNA quantities, multiplied by 10^6. Relative expressions were documented as the logarithm of this value, and represent the mean of at least three independent quantifications. To permit comparison between the values obtained for primer sets ALL1, ALL2, and ALL3, the absolute values obtained for the primer set 1a were added to that for ALL2, because the ALL2 primer set failed to amplify Gp1 RNA (Table 2).The standard deviation (SD) was calculated for each column of logarithmic values.
[a]This value is an outlier and was not included in the calculation for standard deviation (SD).
[b]Values for specimen AA were obtained only once, so the SEM was not calculated.

There were exceptions to this pattern, suggesting that other properties encoded in the sequence, besides the color of emission, might have segregated in a manner consistent with the phylogenetic tree. The tree topology we generated is slightly different from those presented by Ugalde et al., (Ugalde et al., 2004), and Kelmanson and Matz (Kelmanson and Matz, 2003) in polarity. The Ugalde et al., study (Ugalde et al., 2004) suggests that the GFPs are ancestral, or basal to all other fluorescent proteins for *Montastraea*, with blue and red as more derived, while Kelmanson and Matz suggest that cyan fluorescent proteins are basal (Kelmanson and Matz, 2003). Our tree suggests that red is basal and that

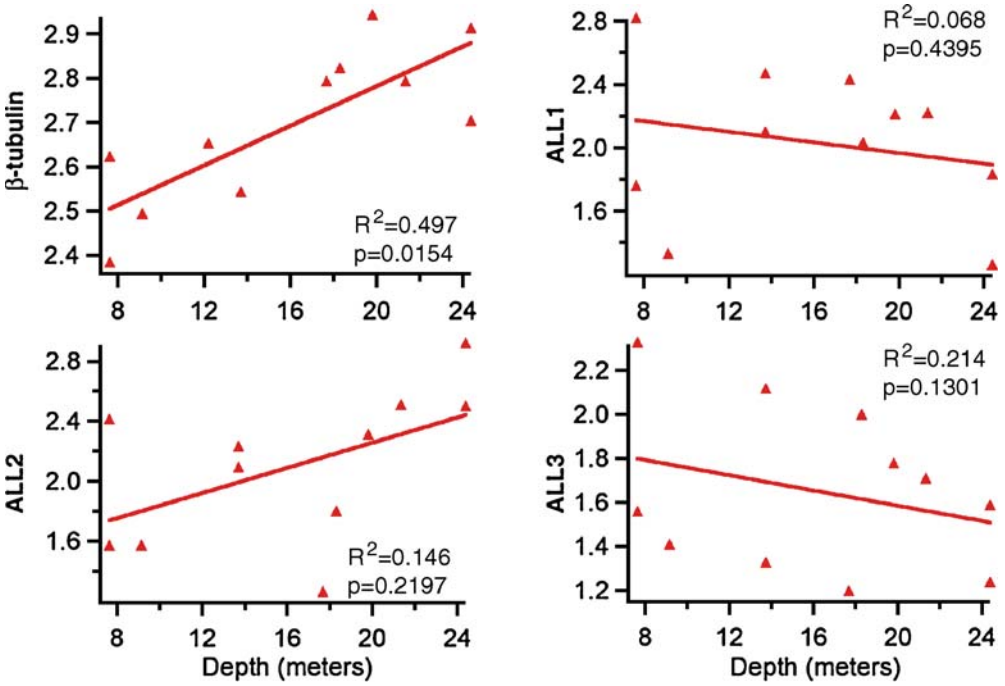

Figure 5. Correlation between depth and relative expression of β-tubulin and all fluorescent proteins. Relative expression values were plotted against depth using data obtained from each specimen of *Montastraea cavernosa*. Relative expression values were documented as the logarithm of the absolute quantity of RNA, normalized to 18S rRNA, and multiplied by 10^6 (Materials and Methods).

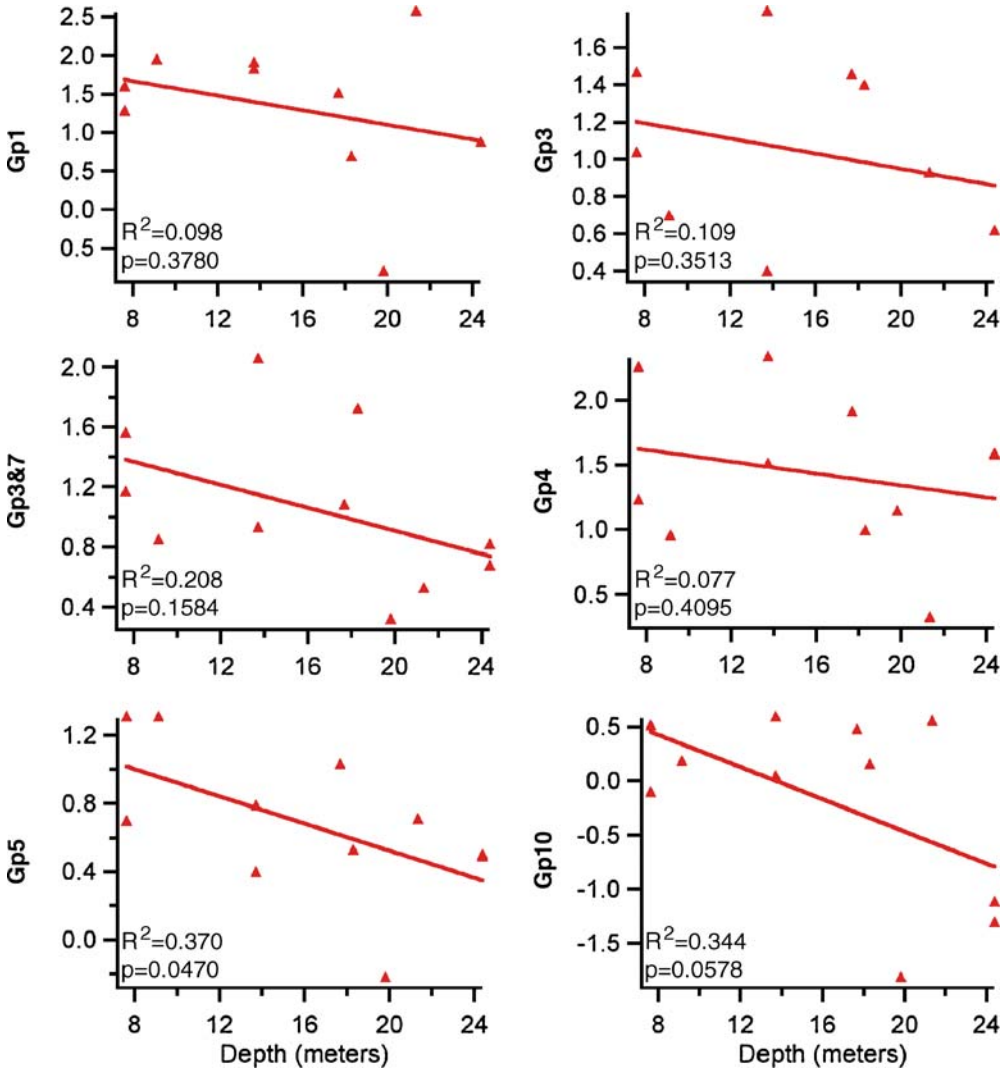

Figure 6. Correlation between depth and relative expression of individual fluorescent proteins. Relative expression values were plotted against depth using data obtained from each specimen of *Montastraea cavernosa*. Relative expression values were documented as the logarithm of the absolute quantity of RNA, normalized to 18S rRNA, and multiplied by 10^6 (Materials and Methods).

green and cyan are derived. This could be due to rooting problems in either study.

We used quantitative PCR to examine the levels of fluorescent protein mRNA in different specimens of *Montastraea cavernosa*. All the samples examined in this report were healthy with no signs of bleaching, necrosis or infection. Given the high sequence identity of these proteins, it would have been difficult to generate specific antibodies that could differentiate the 10 known sequences. Relative to β-tubulin expression, the overall expression of fluorescent proteins varied greatly from specimen to specimen. When converted to absolute quantities, a 10-fold range of expression was observed, suggesting that transcription of fluorescent proteins was highly regulated in different specimens at a particular reef location.

We also examined the combined relative expression of all fluorescent proteins in relationship to depth, but found no correlation between these two parameters. These results are in agreement with a previous report that used Western blot analyses to quantitate all fluorescent proteins in *Montastraea cavernosa* (Mazel et al., 2003).The antibody used in that study recognized most, if not all, fluorescent proteins expressed in *Montastraea cavernosa*, but no effort was made to quantitate the levels of individual fluorescent proteins. Because fluorescent proteins possess distinct spectral properties (Figure 2), it is conceivable that individual proteins could display regulated expression with depth. We therefore sought methods to distinguish the expression of individual fluorescent proteins. Given the high level of sequence identity between the different fluores-

Table 4. Correlation of expression levels obtained with different primer sets

	1a	1b	1&2	3a	3b	4	5	10
1a		0.0726	0.0562	0.8205	0.4937	0.9873	0.0236	0.0022
1b			0.0003	0.5396	0.5460	0.7655	0.3249	0.0447
1&2				0.7986	0.9946	0.5186	0.2806	0.0207
3a					0.0038	0.9731	0.2525	0.3100
3b						0.4069	0.1815	0.1430
4							0.6745	0.7228
5								0.0203
10								

Pairwise correlations of expression levels obtained with different primer sets were performed using Statview. *P*-values are tabulated for each pairwise analysis (highlighted if $P<0.05$).

cent proteins, specific amplification could be achieved in only 5 out of the 10 different sequences. A negative correlation of specific fluorescent proteins was observed with depth, most notably, with Gp5 and possibly 10. These proteins did not have any obvious novel characteristics that might explain this correlation. One protein whose expression could have varied with depth is Gp4, a Kaeda-type fluorescent protein that undergoes an irreversible post-translational change in response to UV exposure, converting its emission spectrum from green to red. Because shallow corals have more exposure to sustained UV illumination than deeper coral, Kaeda-type proteins might be expected to be show higher levels of expression closer to the surface. However, we did not observed such a relationship with Gp4, the Kaeda-type protein cloned from *Montastraea cavernosa*. In addition, we did not observe a relationship between coral coloration (as seen in white light flash photographs) and their depth, suggesting that fluorescent proteins do not contribute to coral color with depth. We therefore speculate that there are other properties of these proteins (Gps 5 and 10), not yet identified, that account for their decreased expression with depth.

Although we could not quantify all individual RNAs using our primer sets, patterns of gene expression emerged after analyses of pairwise expression correlations. We observed that some fluorescent proteins seemed to be coordinately expressed, suggest-

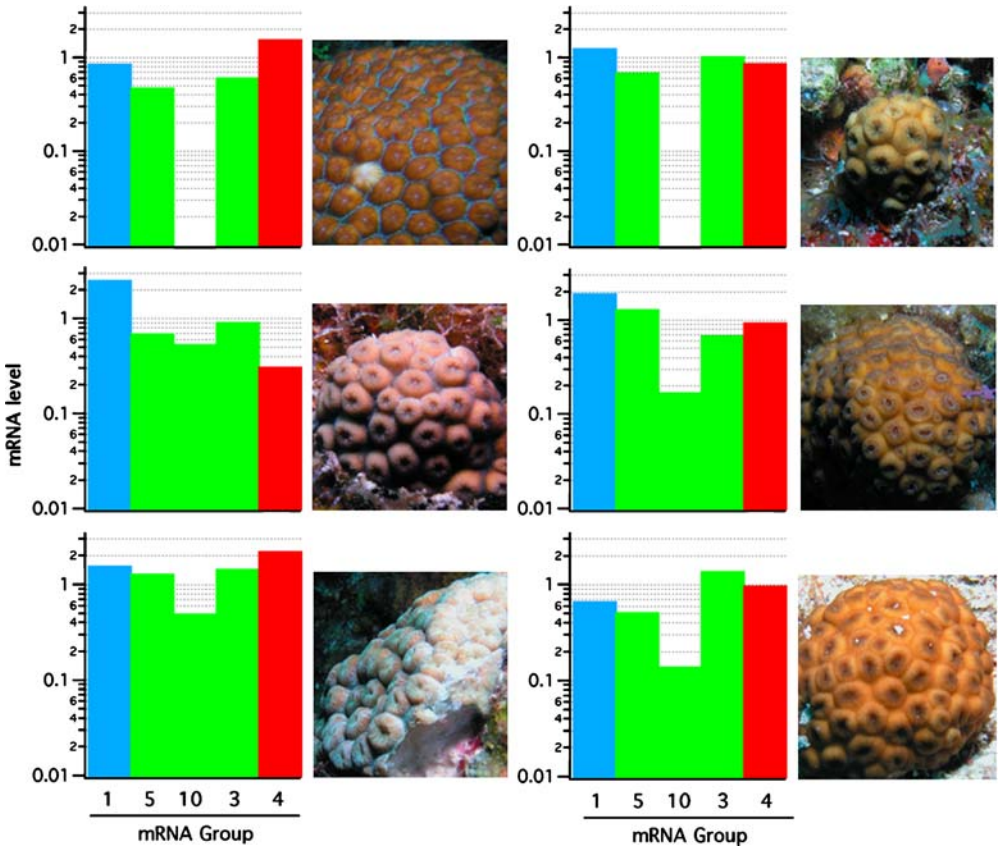

Figure 7. Lack of correlation between the color of coral and the level of fluorescent protein expression. The perceived colors of six coral specimens under natural lighting (photo) are compared to the expression levels of mRNAs corresponding to three color groups: Gp1 = cyan protein; Gp5, Gp10, and Gp3 = green proteins; Gp4 = red protein. The expression scale is logarithmic.

ing that they may be regulated by similar or identical promoters. In particular, there appears to be a consistent positive correlation between the expression of Gps 1, 5, and 10 RNAs. Both Gps 5 and 10 showed a negative correlation with depth, but Gp1 did not. Our sample size is small, and may not have the statistical power to reveal slight correlations with depth, which may have occurred with Gp1, a cyan fluorescent protein. In contrast, we observed that some proteins did not show correlated expression with other proteins. For example, Gp3 RNA did not share coordinate expression with Gps 1, 5, and 10, but may be coordinately expressed with Gp7. Gp4, the sole Kaede-like fluorescent protein in this family, showed no coordinate regulation with any other protein.

These findings could shed light on prevailing hypotheses regarding the function of fluorescent proteins. One study noted more fluorescent corals in shallow waters, and that the presence of fluorescent proteins reduced photoinhibition, leading to the hypothesis that fluorescent proteins are photoprotective for coral (Salih et al., 2000). Because seawater strongly absorbs ultraviolet radiation (Falkowski and Raven, 1997), reduced expression of fluorescent proteins might be expected with depth. We found the expression of some, but not all, proteins to be negatively correlated with depth. Another hypothesis suggests that fluorescent proteins, particularly green ones, serves as "beacons" for symbionts (Hollingsworth et al., 2005). In support of this, it was observed that motile zooxanthellae swarm toward green light (510 to 550 nm), while there was only limited migration toward blue light (460 to 490 nm) (Hollingsworth et al., 2005). If GFP was acting solely to attract symbionts, increased expression would be expected with depth to compensate for the decrease in light. Yet, for most GFPs, we did not observe any correlation of expression with depth.

Another interesting and surprising observation is that, despite the relatively stable expression of β-tubulin, there was a strong positive correlation of its expression with water depth.β-tubulin is often used as a "housekeeping" gene for normalizing expression, but clearly, its expression also changes in response to the environment. We observed a fourfold increase in β-tubulin expression from 7.6 to 24.4 m of water depth. To our knowledge, there have been no reports of transcriptional change for a gene in any organism with depth. As depth increases, so does water pressure. It is conceivable that the increase in β-tubulin, a structural gene, compensates for the adverse effects of increased water pressure.

It was previously reported that the natural coloration of coral was largely due to the presence of fluorescent proteins (Kelmanson and Matz 2003). However, the perceived color of a coral expressing fluorescent proteins depends on many factors, including its repertoire of fluorescent proteins, the ambient lighting spectra, and the quantity and pigments of symbiont algae. The fluorescent component of the color perceived is due to the sum of the fluorescent proteins expressed in a specimen of *Montastraea cavernosa*. We found at least 10 different proteins expressed in our collection of specimens, ranging in color from cyan to red. However, we did not observe any correlation between natural color and the expression of a subset of these fluorescent proteins (Figure 7). The redness in some of the samples may be partly due to the expression of Gp4 protein, which requires exposure to UV light before it converts from green to red. However, this red contribution is dependent on Gp4 protein production, protein turnover, and UV illumination (which is strongly attenuated by depth). This combination of factors probably account for the lack of correlation between the expression of fluorescent proteins of a specified emission, and the perceived color of the coral.

This initial quantification of fluorescent protein expression serves as a step toward gaining a better understanding of the complex natural function of these proteins. While the function of these proteins in non-bioluminescent organisms is still an open question, our study shows that a single coral species can contain multiple fluorescent proteins with different transcriptional regulation. This leads us to conclude that fluorescent proteins may have more varied and dynamic functions than previously suspected.

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