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Novel fluorescent protein from *Hydnophora rigida* possesses green emission

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ABSTRACT

Fluorescent proteins are a family of proteins capable of producing fluorescence at various specific wavelengths of ultra violet light. We have previously reported the identification and characterization of a novel cyan fluorescent protein (HriCFP) from a reef coral species, *Hydnophora rigida*. In search of new members of the diverse family of fluorescent proteins, here we report a new green fluorescent protein (HriGFP) from *H. rigida*. HriGFP was identified, cloned, expressed in *Escherichia coli* and purified to homogeneity by metal affinity and size exclusion chromatography. The dynamic light scattering and gel filtration experiments suggested the presence of monomers in solution. The peptide mass fingerprint on the purified protein established the identity of HriGFP. HriGFP had excitation peak at 507 nm and emission peak at 527 nm. HriGFP was similar to HriCFP except the last 16 amino acid sequence at the C-terminal; however, they have shown least similarity with other known fluorescent proteins. Moreover the computational model suggests that HriGFP is a globular protein which consists of 6 α-helices and 3 β-sheets. Taken together our results suggested that HriGFP is a novel naturally occurring fluorescent protein that exists as a monomer in solution.

1. Introduction

The genes for many fluorescent proteins (FPs) have been cloned and have become a useful resource for making fusion proteins for *in vivo* and *in vitro* studies [1–3]. FPs are unique as they do not require any enzymatic modification or cofactors for the formation of functional structure [2]. Besides GFP from jellyfish, numerous GFP-like proteins have been isolated from hydrozoans and anthozoans of the phylum Cnidaria [4,5]. The GFP-like coral proteins exhibit a broad spectral diversity ranging from blue, green, yellow and red FPs [5] as well as non-fluorescent chromoproteins [6,7].

The range of newly identified novel fluorescent proteins has enabled researchers to selectively activate individual molecules or groups of molecules and easily track their behavior *in vivo* [8–10]. The wild type FPs are further optimized by amino acids substitutions for producing desired optical and biophysical properties with reduced sensitivity to physical and chemical stress [11–14].

With the increasing trend of using monomeric FPs as optical or fusion tags, a great deal of effort is directed to engineer new reporter classes with novel fluorescent properties. Another approach is to discover such FPs from the natural sources. In order to further extend the existing knowledge related to fluorescent proteins, we describe here the identification, cloning, expression, purification, characterization, sequence analysis and modeling of a novel green fluorescent protein, HriGFP, from *Hydnophora rigida*.

2. Materials and methods

2.1. Cloning of cDNA of new fluorescent protein and its expression

Colonies of *H. rigida* (horn coral) were purchased from local commercial marine aquarium (Herndon, Virginia, USA) and kept frozen (−80 °C), until processed. Pre-weighed (5 g) pieces of frozen tissues were ground manually in clean pre-chilled mortar and pestle (free of RNAases) without thawing as described previously [15]. Briefly, total RNA was obtained (Ambion totally RNA kit) and the cDNA was prepared by following the small scale protocol of Ambion RLM-RACE kit. RNA was ligated with 5′-RACE adaptor and reverse transcription was carried out using polyT-RACE primer at

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42 °C for 1 h [13]. The reaction was terminated by heating at 70 °C for 10 min, chilled on ice for 2–3 min and treated with RNase H for 20 min at 37 °C. Partially Nested PCR reaction was carried out using reverse primers targeting the conserved 3′ end of *Montastraea cavernosa* clones (AY181552, AY181556, AF168423, AY056460 and AY037770) [13] (Carter et al., 2004) in combination with RACE 5′-outer adaptor primer. The amplified product of ⩾500 bps was obtained further targeting the conserved 3′ end of *M. cavernosa* clones (AY181552 and AY037770) using 3′-R1mc1 CTTTTTGTGGTCTTGGCTTTTC and RACE 5′-inner adaptor primer. The thermo-cycling conditions were the same as described previously [15]. The amplicon was purified from the low melting agarose gel using Qiagen gel extraction kit and the DNA obtained was ligated using the TOPO-TA expression vector according to manufacturer's instructions and the resultant plasmid was transformed to TOPO competent *Escherichia coli* cells. Fluorescent proteins were identified by visual screening of libraries for fluorescent cDNA clones expressed in *E. coli*. The fluorescent colonies were visualized by using GFP filters (Magnification 100×) in Leica DM4000/5000 B.

2.2. Expression and purification of HriGFP

The open reading frame of HriGFP gene was cloned into pET28a+ vector and transformed into *E. coli* BL21 (DE3) cells. The cells were incubated overnight at 37 °C in agar plates containing 25 mg/ml kanamycin. Glycerol stocks were made from a single isolated colony and used for subsequent several small scale expressions to optimize the protocol for high expression of HriGFP.

For large scale protein expression, the previously described method was used [15] with appropriate modifications. Cell pellets expressing HriGFP (induced with 0.15 mM IPTG in the presence of 25 mg/ml kanamycin) were collected by centrifuging at 6000 rpm (JLA 8.1000 fixed Angle Rotor, Beckman Coulter) and resuspended in 50 ml of lyses buffer (50 mM Tris pH 8.0, 400 mM NaCl, 5% (v/v) glycerol, TritonX 100, 5 mM Imidazole, 1 mM PMSF). Cells were lysed by sonication followed by centrifugation at 18,000 rpm (JA-20 Fixed Angle Rotor, Beckman Coulter) for 30 min at 4 °C. The resultant supernatant was mixed with 2 ml of Ni^{+2}-NTA (Qiagen) resin, pre-equilibrated with lyses buffer and kept for binding at 4 °C. After 1 h, the Ni^{+2}-NTA resin was washed three times with 10 ml of binding buffer containing 30 mM Imidazole followed by elution of HriGFP with 10 ml elution buffer containing 600 mM Imidazole and different fractions were analyzed on a 12% SDS PAGE gel.

The eluted protein from Ni^{+2}-NTA affinity chromatography was further purified through the Hiload™ 16/60 Superdex™ prep grade (Amersham Biosciences, Sweden) pre-equilibrated in a gel filtration column with a buffer containing 20 mM Tris (pH = 8.0), 100 mM NaCl and 5% (v/v) glycerol. All the chromatographic purification was performed at 4 °C using the AKTA FPLC.

2.3. Characterization

2.3.1. Dynamic light scattering (DLS)

DLS experiment using a DynaPro instrument (Protein Solutions, USA) was performed to analyze the homogeneity of all the fractions eluted from gel filtration column at 25 °C with various protein concentrations. The hydrodynamic parameters of HriGFP were calculated by the software provided by the manufacturer.

2.4. Peptide mass finger print (PMF)

Coomassie-stained SDS–PAGE band containing pure HriGFP was subjected to the peptide mass finger print experiment in order to confirm its identity. The steps involved in peptide mass fingerprint include (A) In-gel reduction/alkylation, (B) in-gel digestion, (C) sample cleanup and concentration and finally (D) MS and MS/MS sequencing of the protein digest. The proteins were identified using Mascot program (http://www.matrixscience.com).

2.5. Fluorescence spectroscopic analysis

The fluorescence characteristics of the *E. coli* expressing the green fluorescent protein were measured using total luminescence spectroscopy (JY Horiba Fluoromax-3 spectrofluorometer). 30 absorption scans at an excitation range of 300–600 nm at 10-nm increments and 480 emission scans at an emission range of 320–800 nm at 1-nm increments were obtained for each TLS spectra. The fluorescence intensity measurements are recorded in photon counts per second (cps). Two-dimensional emission scans were measured following TLS measurements as explained previously [15]. In order to minimize second-order harmonics, each emission scan was terminated at a wavelength less than twice the excitation wavelength. Readings were adjusted for the least background fluorescence within a glycerol aqueous medium.

2.6. Sequence analysis and homology modeling of HriGFP

The online tools such as NCBI BLAST and Cobalt RID (seq) were used to identify the protein sequences homologous to HriGFP. Phylogenetic tree based on amino acid sequences of HriGFP and other FPs was developed with the help of MEGA5 tool. The *Aequorea victoria* (AviGFP), *M cavernosa* (McaGFP), and *H. rigida* (HriCFP and HriGFP) sequences were aligned using CLUSTALW and manual positioning in order to identify HriGFP fluorophore.

The structure of HriGFP was modeled on Phyre 2 (Protein Homology/Analogy Recognition Engine) threading server in the intensive mode for fold-recognition and model building. HriGFP structure was modeled on multiple templates with a confidence value of up to 90.3%. Best available multiple templates showing highest similarities with HriGFP were used for homology modeling (templates are shown in Supplementary Table 1).

3. Results

3.1. Identification, cloning of cDNA of new fluorescent proteins and visualization

We identified green fluorescence producing colonies from the library of cDNAs of *H. rigida* using fluorescence microscope. Approximately 50 clones of *H. rigida* cDNA prepared in this study were screened for FPs. The fluorescing colonies of *E. coli* were selected for sequencing of the corresponding plasmids. In the library of cDNAs, we identified fluorescent clones (i.e. clones 1-2, 2-2, 2-3, 2-4, 2-8, 2-11) which were positive for a GFP-like protein and corresponding plasmids were sequenced (Fig. 1). The putative sequence of the clone has been submitted to GenBank (accession number EU408474.1).

The emission and excitation spectra of GFP-like proteins were measured at range of wavelengths. Clones positive for a GFP-like protein had a green centroid with one major excitation peak at 507 nm and emission peak at 527 nm (Supplementary Fig. 1A). We called these clones as HriGFPs.

3.2. Expression and characterization of HriGFP

HriGFP was expressed in *E. coli* and *Bacillus subtilis*. The best condition identified for getting the maximum expression level of HriGFP in *E. coli* (Supplementary Fig. 1B) was 0.15 mM IPTG at 16 °C temperature for 16 h of incubation as reported for HriCFP

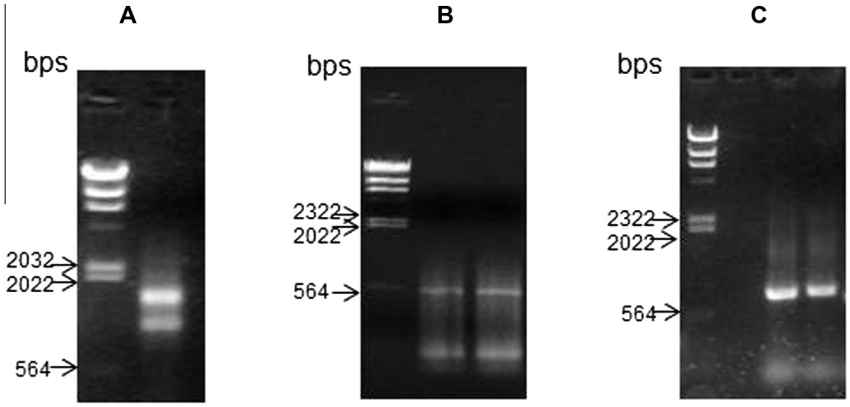

Fig. 1. (A) Total RNA obtained using Ambion totally RNA isolation kit sample. (B) Amplified DNA purified from the low melting agarose gel used for cloning. (C) HriGFP clones: PCR fluorescent DNA products amplified using forward M13 and reverse M13 primers cloned in TOPO-TA expression vector.

[15]. The protein was initially purified using Ni-NTA beads. The SDS gel on the eluted protein from the Ni^{2+}-NTA beads showed some impurities and subsequently a gel filtration chromatography was used to improve the homogeneity of HriGFP (Fig. 2A). The gel filtration profile suggests that HriGFP elutes as the monomer (Fig. 2B).

The dynamic light scattering (DLS) experiments showed that the purified HriGFP was a homogenous species in solution at relatively low concentration (<6 mg/ml), whereas it aggregates as concentration further increases at the given conditions.

The peptide mass fingerprint search in primary sequence data base (NCBI) using the Mascot search engine (Matrix Science) shows the highest score with HriGFP (Fig. 3A and B) and confirmed that the protein cloned, purified and characterized here is the HriGFP.

3.3. Sequence analysis and homology modeling

Results of multiple sequence alignments of protein sequences using NCBI BLAST and Cobalt RID (Seqs) indicated that HriGFP has highest similarity with HriCFP (90%) followed by Hypothetical protein ACD (GenBank Acc: EKD33383.1) (50%), Putative uncharacterized protein (CCY00305.1) (42%), Hypothetical protein (YP_007918.1) (30%), UvrABC system protein C (YP_004446894.1) (31%), UvrABC system protein C (YP_004253888.1) (30%), Excinuclease ABC subunit C (YP_005508972.1) (30%), Excinuclease ABC subunit C (YP_003389618.1) (30%), UvrABC system protein C (27%), Excinuclease ABC subunit C (WP_009137569.1) (27%) and hypothetical protein (WP_019894762.1) (26%). However, the search against the pdb database showed only UvrABC system protein C (Supplementary Table 1) is the only closest homologue structurally characterized. Phyre 2 in intensive mode also indicated UvrABC system protein C as the best available template for homology modeling. Sequence similarities are shown in Supplementary Fig. 2.

It is apparent from phylogenetic trees that *H. rigida* fluorescent proteins (HriGFP and HriCFP) are phylogenetically unrelated to any other FP. HriGFP and HriCFP make a distinct out-group and branch length of about 100 timelines unit shows that there is only little or no similarity between the *H. rigida* FPs and other Anthozoan FPs (Supplementary Fig. 3).

The predicted structure of HriGFP comprised of three β sheets (β1↑, β2↓, β3↑) flanked by one α helix (α1) at N terminus and three α helices (α4, α5, α6) at C terminus (Fig. 4A and B) while α2 and α3 located between β2↓ and β3↑ whereas putative fluorophore, KYG, is present at C terminus of α2 (Fig. 4C and D).

4. Discussion

Previously several distinct FPs with different spectral properties have been isolated from a single Anthozoa species [16,17]. From

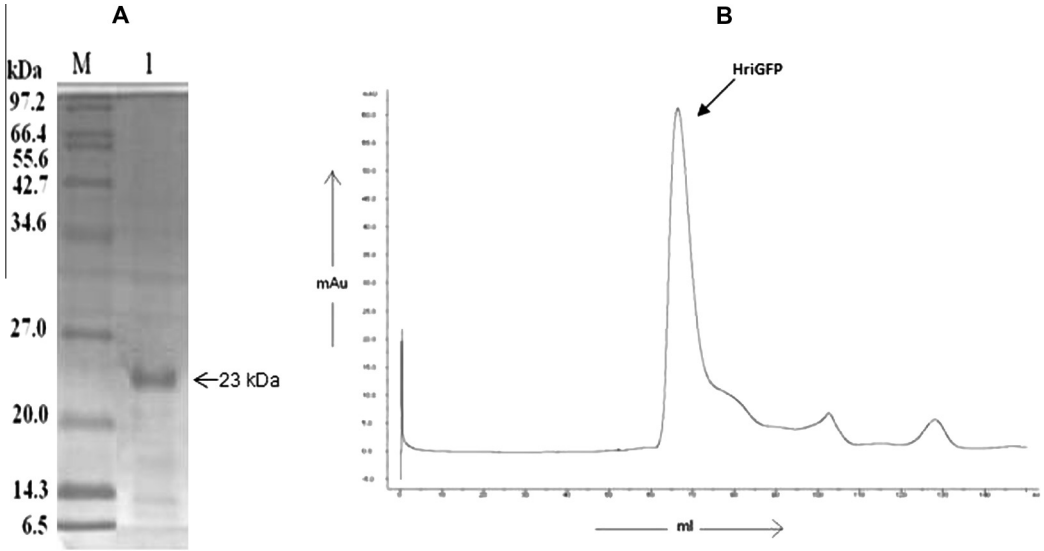

Fig. 2. HriGFP purification. (A) HriGFP expression and purification after gel filtration chromatography. Lane 1 – Marker, Lane 2 – purified HriGFP. (B) Gel filtration chromatography of HriGFP. Elution profile shows that HriGFP elutes as the monomer.

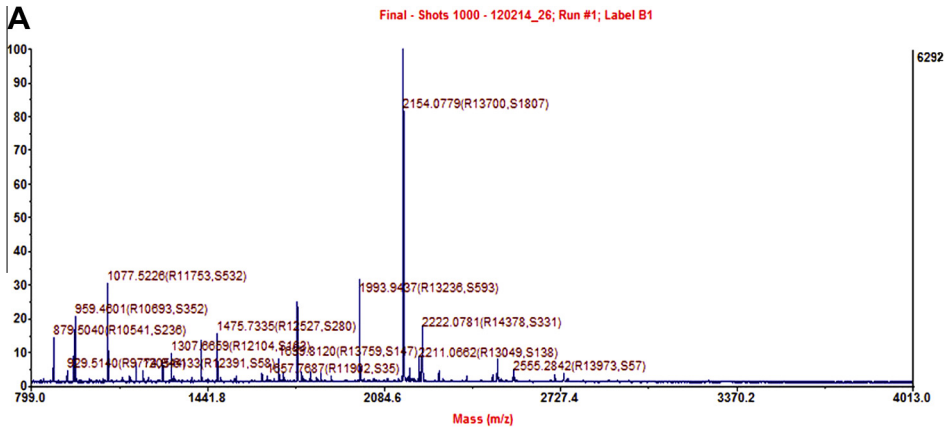

Fig. 3. (A) Mass spectrum of tryptic peptides digest of HriGFP. Spectra were recorded in a MALDI TOF/TOF in reflection mode. MS/MS of selected peptides were determined by LIFT. The protein identity was established by a mass fingerprint search in primary sequence database (NCBI) using the Mascot search engine. (B) Tryptic digested peptide that shows match with HriGFP protein in NCBI database as shown in bold underline.

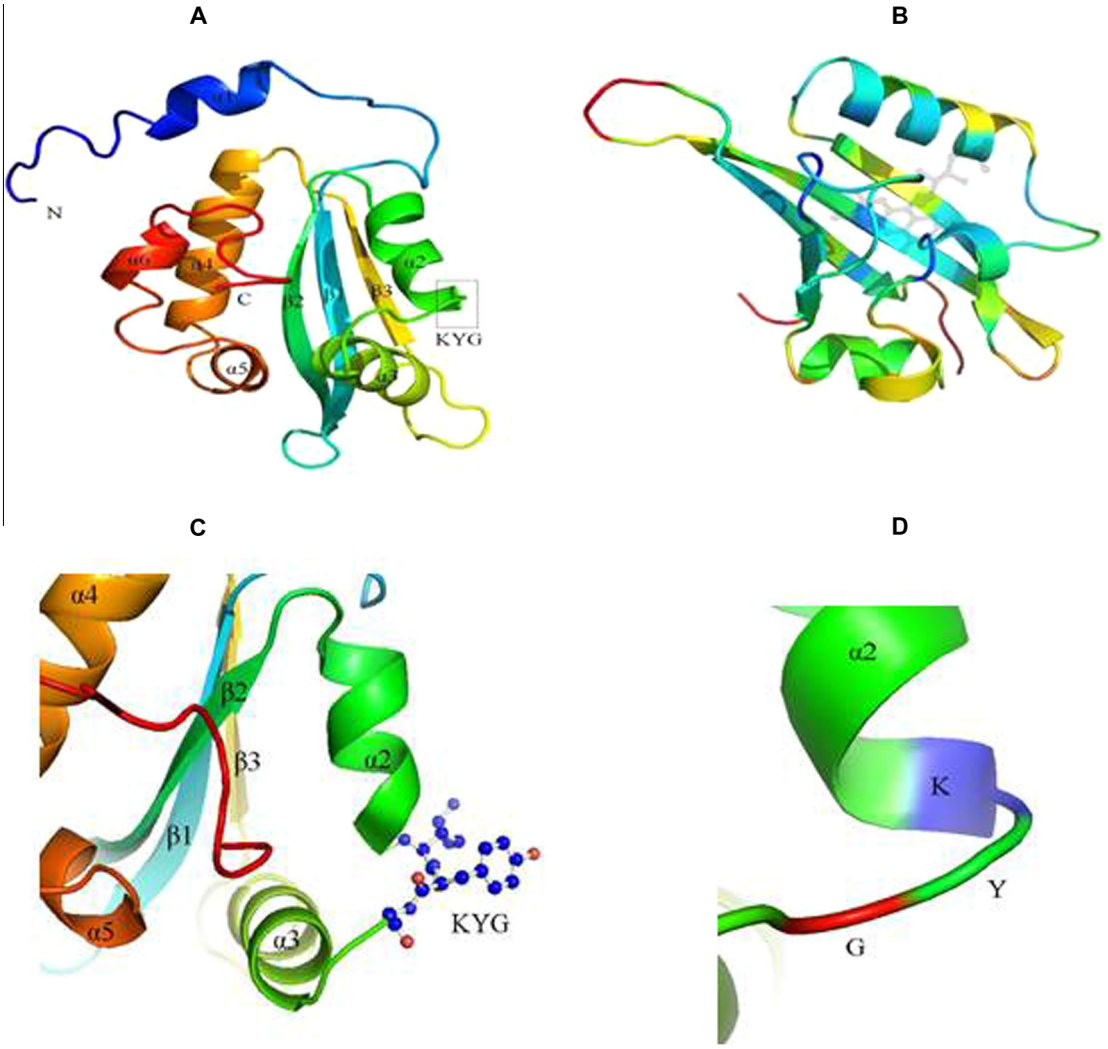

Fig. 4. (A): The structure of HriGFP predicted by using Phyre 2 in intensive mode. The HriGFP structure is strikingly similar to the structure of Arabidopsis photoprotein iLOV (PDB ID: 4EES). (B) Christie et al. [20]. (C, D): Location of fluorophore residues KYG in HriGFP structure, K is the part of α2, while Y and G residues are located next to K residue between α2 and α3.

our previous study HriCFP [15] and HriGFP from our current study isolated from *H. rigida* are also phylogenetically distinct from other known FPs (Supplementary Fig. 3).

Fluorescent proteins are generally non-toxic so they can be expressed in different organisms with little or no effect on the host cell physiology [1]. The cloned sequence of HriGFP contains the endogenous coral promoter along with its ORF and was expressed independent of the *LacZ* operon. We have confirmed it by sub-cloning the insert into a shuttle vector pHIS1522 (MoBiTec, Goettingen, Germany) and expressing the protein in *Bacillus megaterium* and demonstrated that these constructs were also fluorescent (data not shown).

GFP of *A. victoria* and most of other FPs have a β barrel structure whereas the recently solved structures of some of the FPs have shown absence of β barrel. Some of the examples of FPs without β barrel structure include UnaG (PDB ID: 413B) a fluorescent protein from muscles of Japanese eel [18], green fluorescent protein (PDB ID: 2PSF) from *Renilla reniformis* [19] and a small fluorescent protein (115 amino acid residues) called iLOV (PDB ID: 4EES) [20]. These non- β barrel FPs along with our non-β barrel structure of HriGFP suggest that proteins can exhibit fluorescent properties even in the absence of a β barrel structure. These alternative structures may prove to be valuable guiding models for building small sized artificial FPs for biotechnology applications. Interestingly, the homology based predicted structure of HriGFP have shown resemblance with the known structure of a fluorescent protein iLOV (Fig. 4A and B).

Fluorophore of FPs is responsible for their fluorescence and is usually formed spontaneously from an indigenous tri-peptide motif of FPs. The reported fluorophore in the *M. cavernosa* GFP is a QYG motif [2] whereas amino acid sequence comparisons indicated a putative KYG fluorophore motif for HriGFP (Supplementary Fig. 4) which is the same as for HriCFP [15]. The fluorescence excitation and emission maxima of HriGFP (507 and 527 nm, respectively) were unique, when compared to those of 490 and 509 nm for enhanced GFP (GFPmut1) [21], 395 nm and 508 nm of wild type GFP and 450 nm and 495 nm for HriCFP [2,15]. The computationally modeled structure of HriGFP revealed that its putative fluorophore motif (KYG) was found to be located in the outer part of the protein (present at the C terminus of an α helix (α2) (Fig. 4C and D). The location illustrated hydrophilic nature of fluorophore which is totally opposite as compared to the fluorescence proteins bearing β barrel structures (which have their fluorophore located in the internal core surrounded by β barrel).

It is previously noted that FMN (flavin mononucleotide) binding FPs produce cyan to green fluorescence [22] which is the same as observed in HriCFP (cyan) and HriGFP (green). Because FMN is naturally present in all prokaryotic and eukaryotic cells [23], we cannot exclude the possibility of interaction of HriGFP with FMN in studied bacterial host cells. Thus whether FMN has any role in the fluorescent properties of HriGFP needs to be clarified in future studies.

Fluorescent proteins from anthozoan and hydrozoans tend to oligomerize and hence are avoided to be used in constructing fusion proteins, however, naturally occurring rare monomeric FPs [24,25] and HriGFP can be useful alternates. In many cases the fluorescent marker protein needs to be monomeric for correct localization of the fusion partner [26,27] and relatively small size (<150 amino acids) is therefore a desirable feature for generating fluorescently labeled fusion proteins with small imprints [28,29].

HriGFP due to its smaller size like HriCFP may prove a superior tag and moreover pair can also act as ideal candidate with their distinguishable excitation and emission wavelengths. Thus HriGFP is a novel green fluorescent protein and appears to be a member of non-β barrel FPs family with some similarity to iLOV and related FMN binding FPs. However, further studies will shed light on any similarities of functions between HriGFP and iLOV domains in order to understand the physiological functions of HriGFP in corals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.042.

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