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Bioluminescence of *Aequorea macrodactyla,* a Common Jellyfish Species in the East China Sea

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Abstract: Studies of the bioluminescent mechanisms of jellyfish have been mainly confined to one species, *Aequorea victoria.* We describe the luminescent system of another species, *Aequorea macrodactyla,* which is commonly found in the warmer waters on the coastal region of East China Sea. The luminescent system of this species consists of a green fluorescent protein (GFP) and one or more aequorins. The GFP gene is 1042 bp. It encompasses a coding sequence of 717 bp organized as 3 exons, and it is predicted to specify a 27-kDa peptide, which shares 80% amino acid sequence identity with the GFP of *A. victoria.* The entire coding sequence was cloned into the pTO-T7 expression vector and expressed in *Escherichia coli.* Compared with GFP of *A. victoria,* the purified expressed protein exhibited an excitation peak at a higher wavelength of 476 nm and an emission peak at a lower wavelength of 496 nm, with a higher quantum yield of 1.0. The other photoprotein, aequorin, is encoded in a single open reading frame of 585 bp specifying a 23-kDa apoprotein. The gene was cloned in to the same expression vector and expressed in *E. coli.* The activity of the photoprotein was reconstituted by incubating the expressed apoprotein with coelenterazine f. In the presence of Ca^{2+} the reconstituted aequorin exhibits an emission peak at 470 nm. The kinetics of regeneration and the photoactivities of the reconstituted aequorins of the 2 species of jellyfish are similar. Nevertheless, *Aequorea macrodactyla* is expected to appear brighter and more "blue" than *Aequorea victorea* because of the differences in the photoactivity of their GFPs.

Key words: bioluminescence, jellyfish, *Aequorea macrodactyla,* East China Sea.

INTRODUCTION

Bioluminescence is a common feature among marine organisms including jellyfish. Previous studies showed that the luminescent system of a species of jellyfish, *Aequorea victoria,* consists of a 27-kDa green fluorescent protein (GFP) and the aequorin complex. The latter is made up of a 22-kDa apoaequorin, the luminophor coelenterazine, and molecular oxygen. Upon binding of calcium ions, coelenterazine is oxidized to coelenteramide releasing carbon dioxide and emits blue light with a maximal emission at 469 nm. The energy generated from activation of aequorin in turn serves to activate GFP causing it to emit a green light with a maximal emission at 508 nm (Ward et al., 1980).

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GFP has been widely used as a reporter gene in the study of gene expression in microbial, plant, insect, and mammalian cells (Chalfie et al., 1994; Barthmaier and Fyrbery, 1995; Hu and Cheng, 1995; Scales et al., 1997; Straight et al., 1997). The Ca-dependent emission from aequorin has rendered the photoprotein useful for measuring the intracellular Ca^{2+} concentration (Cessna et al., 1998; Hampton et al., 1998; Leung et al., 1998; Sala-Newby et al., 1998).

This report describes the luminescent system of another species of jellyfish, *Aequorea macrodacytyla,* which is commonly found in the warm coastal waters of the East China Sea (Zheng et al., 1984). The results of this study showed that while the general features of the luminescent system of this species were similar to that of *Aequorea victoria,* the GFPs of the 2 species exhibited distinct photoactivities.

Materials and Methods

Extraction of Nucleic Acids from *Aequorea macrodactyla*

Jellyfish were collected from the coastal region of East China Sea near Xiamen. The outer margins of 2 of the organisms were ground in a homogenizer with 4 ml of DNA extraction buffer (20 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 1% sodium dodecylsulfate [SDS]). The homogenate was extracted with an equal volume of phenol, the upper aqueous phase was aspirated and digested with 5 µl of RNase A (10 mg/ml) at 37°C for 2 hours, and the mixture was extracted again with phenol. The extracted DNA contained in 0.1 M sodium acetate (pH 5), was precipitated with a double volume of absolute ethanol, and was dissolved in 10 mmol/L Tris-HCl, pH 7.5, 1.0 mmol/L EDTA, to give a concentration of 0.1 mg/ml, and was stored at −20°C.

For the extraction of RNA, 2 outer margins were ground in a homogenizer with 3 ml of Trizol at 4°C, and the homogenate was extracted with 600 µl of chloroform. RNA contained in the aqueous phase was precipitated with an equal volume of isopropanol dissolved in 1 × RT buffer to a final volume of 40 µl and stores at −80°C until use. RNA was converted to complementary DNA with avian myeloblastosis virus reverse transcriptase and random primers.

Isolation and cloning of GFP and *Aequorin* Genes

The luminescent genes of *A. macrodactyla* were amplified from the extracted DNA or cDNA using the primer pairs as indicated in Table 1. The resulting polymerase chain reaction (PCR) products were analyzed by Southern blotting using digoxigenin (DIG)–labeled probes specific for GFP or the aequorin gene. A 900-bp (GFP-900) and a 550-bp (GFP-500) genomic DNA sequence and a 720-bp cDNA sequence (GFPc720) of the GFP gene were identified by the GFP probe, and a 620-bp genomic DNA sequence of the aequorin gene (A620) was identified by the aequorin-specific probe. These sequences were selected and cloned into the pMD-18 T plasmid. The GFP-specific DIG probe was a 331-bp sequence amplified from a cloned GFP gene of *A. victoria* (pGFPUV), and the aequorin-specific DIG probe was a 267-bp sequence amplified from genomic DNA of *Aequorea macrodactyla.*

Expression of GFP and *Aequorin* Genes

The 720-bp GFP cDNA was cut from the plasmid with *Nde*I/*Sal*I, the 620-bp aequorin gene was cut from the plasmid with *Eco*RI/*Xho*I, and the genes were recloned into the expression vector pTO-T7. *Escherichia coli* strains BL21 (DE3) transformed with these genes were grown at 37°C in 200 ml of LB medium (Kan 100 µg/ml) until reaching 0.8 U at optical density 600 nm. IPTG was added to a final concentration of 0.2 mmol/L, and the cultures were further incubated at 15°C for 36 hours. The cells were harvested by centrifugation and resuspended in 10 ml of 20 mmol/L Tris-HCl (pH 7.6). Cell disruption was achieved by sonication at 4°C (Sonics & Materials Inc., Danbury, Conn., Model Sonifier). The sonicates were centrifuged at 15,000 rpm for 10 minutes in a refrigerated centrifuge (Model 3K18, Sigma, St Louis, Mo.). The pellet was dissolved in 10 ml of 20 mmol/L Tris-HCl (pH 7.6). Twenty-five microliters of supernatant and the pellet was analyzed by SDS–polyacrylamide gel electrophoresis staining with Coomassie blue. The concentrations of the stacking gels was 5%, and the resolving gels, 12%. The product was quantificated by UVIBAND software for Windows (Version 99).

Purification of Expressed GFP

GFP contained in the cytosol was precipitated at 50% saturation of ammonium sulfate, dissolved in 1× PBS (pH 7.45), dialyzed in the same buffer, and filtered through 0.22 µM of microporous membrane. The sample was purified by high-performance liquid chromatography (HPLC) (Beckman System Gold Nouveau HPLC 125 NMP/166 NMP) using an SK GEL SW3000 21.5 × 6 column followed by an

Table 1. Primers for Amplification of GFP and Aequorin Genes

	Primers	Products
wgF4	5′–GAT AAC AAA GAT GAG TAA AGG AG–3′	GFP-900
wgR4	5′–ATT AGG AAT GCA CTC CAG TAG–3′	GFP-900
wgF3	5′–GTC ACT ACT TTC TCT TAT GG–3′	GFP-900
wgR5	5′–GTG TCA ATT GGA AGT CTG G–3′	GFP-900
wgF5	5′–CAC AGG ATA ACA AAG ATG–3′	GFP550
wgR2	5′–CAA TTG GAG TCT GGA CAT TTA–3′	GFP550
wgR3	5′–AAA CTT GAC TTC AGC ACG TCA–3′	GFP550
wgF4	5′–GAT AAC AAA GAT GAG TAA AGG AG–3′	cDNA of GFP
wgF6	5′–GGA TCC ATG AGT AAA GGA GAA GAA C–3′	cDNA of GFP
wgR6	5′–TTA TTT GTA TAG TTC ATC CAT GCC–3′	cDNA of GFP
aqF93	5′–GTC TCG ACA ACA ACA AGC AAA C-3′	Aequorin
aqR712	5′–GAG CTT CTT AGG GGA CAG CT–3′	Aequorin

ion exchange column, Q Hyper D 20. The elution buffer used for the gel filtration column was 1× PBS, pH 7.45, and that for the ion exchange column was 0.1 M NaCl/25 mM bis-Tris, pH 6.5, by HPLC.

Analysis of the Fluorescence Properties of the Expressed GFP and Apoaequorin

Excitation and emission spectra of the expressed GFP were measured by fluorescent panorama (Fluorat-02 Panorama, Lumaex, Russia). The fluorescence quantum yield of expressed GFP was determined according to the method described by Cheng et al. (1990) using FITC as the reference.

Aequorin was regenerated from apoaequorin by adding 10 μl of 2-ME and 6 μg of coelenterazine (1 μg/μl in absolute methyl alcohol) to 1.0 ml of the extract, and the mixture was allowed to stand for 3 hours in an ice bath (Shimomura and Johnson, 1975). The mixture was placed in the fluorescent panorama and injected with 1.5 ml of 30 mmol/L $CaCl_2$/30 mmol/L Tris-HCl, pH 7.6.

Analysis of DNA and Amino Acid Sequence

The software, DNASIS, was used to analyze the open reading frames of DNA sequence, and CLUSTALX and DNASTAR were used to analyze the identity of amino acid sequence of protein.

Bacterial Strains and Plasmids

The *E. coli* strains used were JM101 and BL21 (DE3). The plasmids employed were pTO-T7, pTO–T7EGFP (Luo et al. 2000) and pEGFP and pGFPUV were purchased from Clontech (Palo Alto, Calif.).

Enzymes and Chemicals

All restriction endonucleases and *E. coli* T4 DNA ligase were purchased from Promega (Madison, Wis.). NBT/BCIP and gel extraction mini kit were purchased from Watson (Shanghai, China). AMV and random primers were purchased from Takara (Kyoto, Japan). Trizol was purchased from Gibco (Rockville, Md.). DIG Labeling Mix was purchased from Beohringer Mannheim GmbH (Germany). *Taq* enzyme and dNTP were purchased from Sangon (Shanghai, China). FITC was purchased from Promega. Coelenterate f was purchased from Molecular Probes (Eugene, Ore.). The primers used in the present study were as described in Table 1.

GenBank Numbers

The GenBank accession numbers are as follows: AEVGFPA, M62653; AEVGFP, L29345; AVGFP1, X83959; AVGFP2, X83960; AEVAQ440X, L29571; AVEAEQA, M16103; gfpxm, AY013824; and aeqxm, AY013823.

Results

The Luminescent Genes of *A. macrodactyla*

The GFP Gene

The complete genomic sequence of the GFP gene of *A. macrodactyla* was cloned into the pMD-18 T vectors as 2 overlapping fragments of 900 and 550 bp. The former was located between the coordinates 1 and 900 of the genomic sequence, and the latter, between 492 and 1042 (Figure 1). The genomic DNA contains a coding region of 717 bp and it is organized as 3 exons of 206, 295, and 216 bp, respectively. The exons share 76% to 84% sequence identity with the homologous regions of the prototype GFP gene of *A. victoria* (*aeqgfp10*), but the introns of *A. macrodactyla,* of 182 and 139 bp, were markedly smaller than the corresponding regions of the genomic DNA of *A. victoria.*

The entire coding region of the GFP gene (*gfpxm*) of *A. macrodactyla,* contained in a fragment of 720 bp, was am-

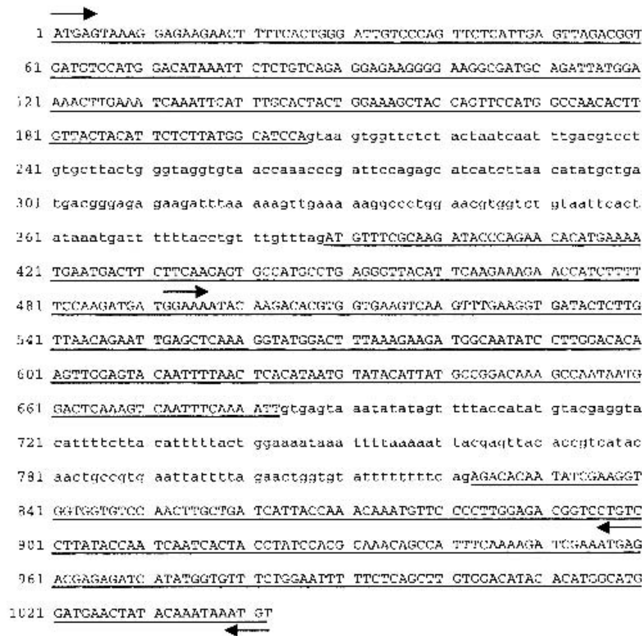

Figure 1. GFP gene of *Aequorea macrodactyla.* The genomic sequence of the GFP gene of *Aequorea macrodactyla* of 1042 bp was cloned as 2 overlapping fragments of 900 bp and 550 bp (*arrows*). The coding sequence of 719 bp is organized into 3 exons of 206, 295, and 216 bp (*underlined*).

Figure 2. Predicted amino acid sequence of GFP of *Aequorea macrodactyla.* The predicted amino acid sequences of GFP of *Aequorea macrodactyla* and *Aequorea victoria* differ by 39 amino acid residues (*boxes*), with 9 of the mismatches being due to nonconservative changes (*asterisks*).

plified from the cDNA of *A. macrodactyla* and cloned into the pMD-18 T vector. It is predicted to encode a 27-kDa peptide, which shares 84% amino acid sequence identity with the peptide encoded by the GFP gene *A. victoria* (Figure 2). The 2 proteins differ by a total of 39 amino acid residues, with 9 of the mismatches being nonconservative changes.

The Aequorin Gene

Aequorin of *A. macrodactyla* was encoded by a single uninterrupted open reading frame of 585 bp. The gene (*aeqxm*) was amplified from the genomic DNA of *A. macrodactyla* and cloned into the pMD-18 T vector as described. It shares 80.7% nucleotide sequence identity with the prototype aequorin gene, *AEVAQ440X,* derived from *A. victoria* (Figure 3). The aequorin gene is predicted to encode a 23-kDa peptide, which shares 84.7% amino acid sequence identity with the peptide encoded by the prototype aequorin gene from *A. victoria.* The 2 proteins differ by 32 amino acid residues, with 9 of the mismatches being due to nonconservative changes.

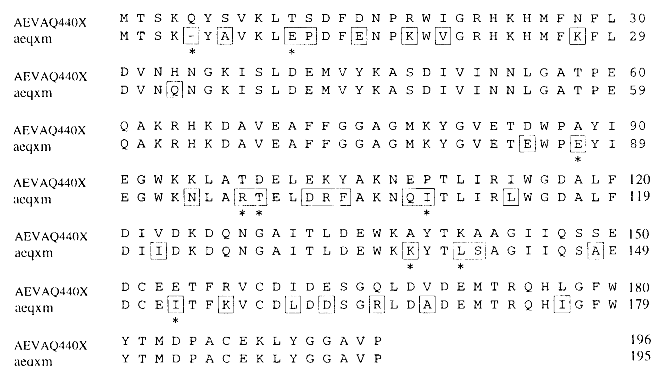

Figure 3. Amino acid sequence of aequorin of *Aequorea macrodactyla.* Aequorin was encoded by a single open reading frame of 585 bp. The predicted amino acid sequence of the photoprotein of *Aequorea macrodactyla* differs from that of *Aquorea victoria* by 32 amino acid residues (*boxes*), with 9 of the mismatches being nonconservative changes (*asterisks*).

Photoproteins of *A. macrodactyla*

Expression of Photoproteins in E. coli

The gfp and aequorin genes of *A. macrodactyla* were cloned into the expression vector, pTO-T7, and expressed in competent *E. coli* cells. Figure 4 shows that the expressed 27-kDa GFP constituted about 50% of the total bacterial protein (lane 2), with 90% being found in the inclusion body (lane 3) and the remaining 10% in the cytosol (lane 4). The expressed 23-kDa apoaequorin was found to constitute about 40% of the total bacterial protein (lane 5), with about

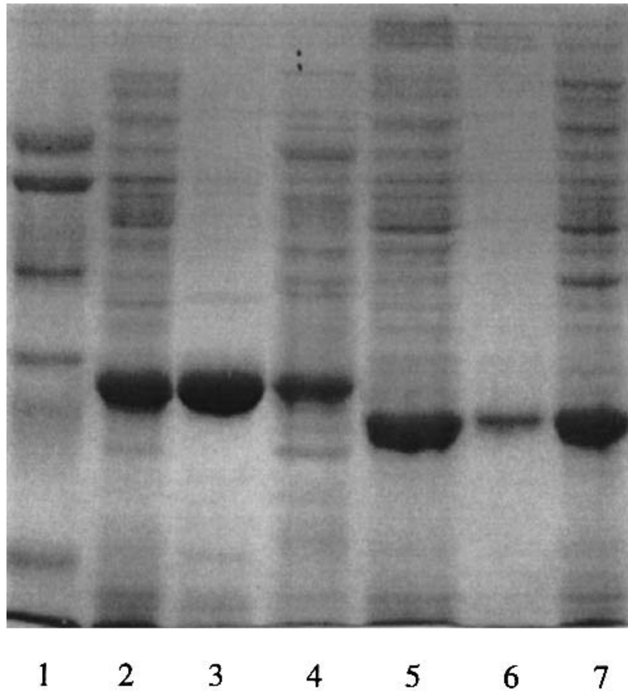

Figure 4. SDS-PAGE of expressed GFP and apoaequorin. The genes encoding the GFP and apoaequorin of *Aequorea macrodactyla* were recloned into the expression vector pTO-T7 and expressed in *E. coli.* The cells were disrupted by sonication and then centrifuged at 15,000 rpm for 30 minutes. Following SDS-PAGE, expressed GFP contained in the sonicates (lane 2), the pellets (lane 3), and the cytosol (lane 4) was identified as a 27-kDa band. The expressed apoaequorin migrating as a 23-kDa band was similarly identified in the sonicate (lane 5), in the pellet (lane 6), and in the cytosol (lane 7).

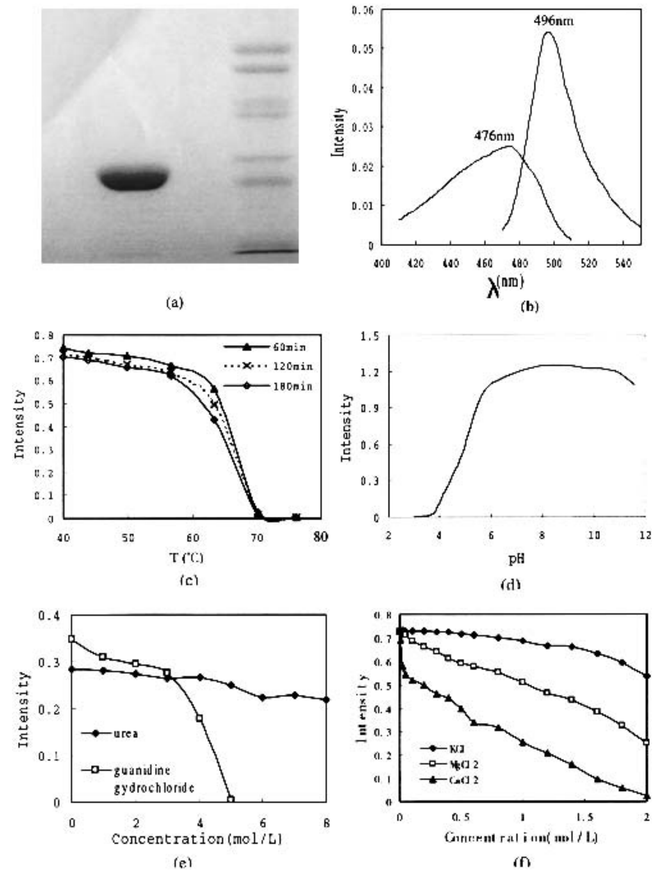

Figure 5. Photoactivity of expressed GFP. **a:** The expressed GFP was purified by HPLC and subject to SDS-PAGE. **b:** The purified GFP exhibited an excitation peak at 476 nm and an emission peak at 496 nm. Emission intensity at 496 nm was measured after purified GFP was heated at 60°C for different times **(c),** and following treatment at different pH values **(d),** different concentrations of urea or guanidine hydrochloride **(e),** or different concentrations of KCl, $MgCl_2$, or $CaCl_2$ **(f).**

80% being found in the cytosol (lane 6). The efficient expression of the photoprotein was attributed to the enhancer contained in the expression vector (Luo et al., 2000).

Photoactivity of Bacterially Expressed GFP

The expressed GFP was purified from the cytosol rather than the inclusion bodies to avoid having to expose the photoprotein to denaturing agents. It was estimated by the intensity of the 27-kDa band in the SDS-PAGE that the protein had been purified to 95% homogeneity (Figure 5, a). The purified product exhibited an excitation peak at 476 nm and an emission peak at 496 nm with a quantum yield of 1 (Figure 5, b). The activity was stable after heating for 1 hour at up to 60°C (Figure 5, c), at pH between 6.0 and 10 (Figure 5, d). The activity of the expressed GFP was also stable to treatment with 4 M guanidine hydrochloride and 8 M urea (Figure 5, e) and divalent cations, especially Mg (Figure 5, f).

Photoactivity of Bacterially Expressed Aequorin

Figure 6 shows that the aequorin complex was regenerated by incubating the expressed apoaequorin in the presence of coelenterazine f. Rapid regeneration of activity occurred within the first 30 minutes, and the activity was further increased, albeit more slowly, upon further incubation for 200 minutes or more (Figure 6, a). Regeneration of the photoprotein was retarded at pH 7.5 or higher (Figure 6, b) and at temperatures of 30°C or higher (Figure 6, c).

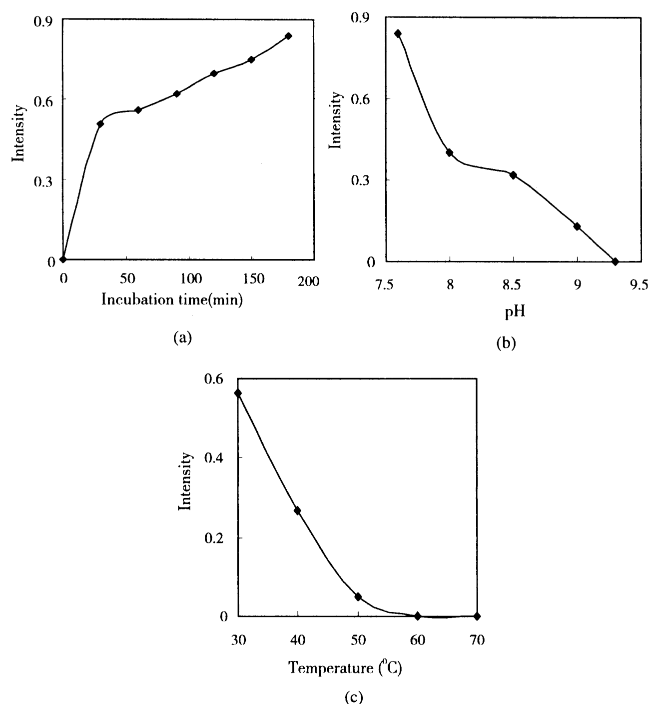

Figure 6. Regeneration of the photoactivity of expressed aequorin. The expressed apoaequorin was incubated with coelenterazine f and 2-ME. Regeneration of photoactivity was measured by the intensity of emission at 470 nm in the presence of Ca at the indicated times (**a**), under the different pH values (**b**), and at the indicated temperatures (**c**).

Discussion

GFP of Two Species of Jellyfish

Aequorea macrodactyla is a species of luminescent jellyfish commonly found in the warm waters on the coast of the East China Sea, which characteristically emits a green light. To study its luminescent system, we have cloned the complete genomic DNA of GFP of 1042 bp in 2 overlapping fragments of 900 and 550 bp, the entire 717 coding sequence for GFP contained in a 720-bp fragment and 585-bp full-length aequorin gene. We have expressed the proteins in *E. coli* and characterized their photoactivity. Cloning was achieved by PCR amplification of the genes directly from DNA or cDNA from the organism. The amplified products were selected by Southern blotting and then cloned into the pMD-18 plasmid. This approach facilitated the work by focusing on the desired products. The expression vector used contained an enhancer, which contributed to the efficient expression of the photoproteins (Luo et al., 2000).

As in the *A. victoria,* the luminescent system of *A. macrodactyla* consisted of 2 proteins, ie., GFP and aequorin. The genomic DNA of GFP of both species was organized as 3 exons, which share 79% to 84% sequence identity, and GFPs specified by these genes are predicted to share 83.6% amino acid sequence identity. The introns of the GFP gene of *A. macrodactyla* were substantially shorter than the corresponding regions of the GFP gene of *A. victoria* (Prasher et al., 1992). GFP of *A. victoria* has a major excitation peak at 395 nm, a minor peak at 475 nm, and an emission peak at 508 nm, with a quantum yield of 0.79 (Patterson et al., 1997). The photoactivity was attributed to activation of a *p*-hydoxy-benzyliden emidazolone chromophore by energy generated from cyclization and oxidation of Ser-Tyr-Gly residues located from position 65 to 67 of the protein (Prasher et al., 1992; Cody et al., 1993). The same Ser-Tyr-Gly residues are conserved in GFP of *A. macrodactyla,* and the protein exhibits a higher excitation peak at 476 nm, a lower emission peak at 496 nm, with a higher quantum yield of 1.0. The distinct photoactivity of GFP may impart to *A. macrodactyla* a slightly bluer and brighter gross appearance than *A. victoria.* The distinct photoactivity is presumably attributable to the difference in the amino acid sequence between GFPs of the 2 species of jellyfish. Indeed, mutagenesis studies have shown that the photoactivity of GFP is influenced by minor changes in amino acid sequences (Heim et al., 1994, 1995; Heim and Tsien, 1996; Mitro et al., 1996).

Aequorin of Two Species of Jellyfish

At least 5 isotypes of aequorins specified by *A. victoria,* presumably encoded by different members of a gene family, were resolved by 2-dimensional electrophoresis (Cormier et al., 1989). The aequorin gene presently cloned from *A. macrodactyla* probably corresponds to one member of such a gene family. The cloned gene consists of a single open reading frame of 585 bp. It is predicted to specify a 23-kDa apoprotein comprising 195 amino acid residues and share 84.7% amino acid sequence identity with an aequorin gene cloned from *A. victoria.* The photoprotein was reconstituted by incubating the bacterially expressed apoaequorin with the chromophore coelenterazine f. In the presence of Ca^{2+} ions, the reconstituted photoprotein emitted blue light at the peak wavelength of 470 nm, similar to the native product from *A. victoria* (Charbonneau et al., 1985; Inouye et al., 1985, 1986; Prasher et al., 1985). In addition, the generation of the active photoprotein from either of the apoaequorins followed similar kinetics and had similar requirements.

Thus, despite their distinct nucleotide and amino acid sequences, and unlike GFP, aequorins specified by the genes isolated from the 2 species of jellyfish share similar photoactivities.

Distinct Bioluminescence

Most studies of bioluminescence of marine jellyfish in the past have focused on *A. victoria.* This is the first description of the luminescence of another species, *A. macrodactyla,* which is commonly found in the coastal regions of the East China Sea. The results showed that GFP and aequorin are major components of *A. macrodactyla,* and both of the proteins are genetically and structurally different from those of *A. victoria.* Moreover, whereas the photoactivity of aequorin was similar in both species, the activity of GFP was distinct in the 2 species, and according to the difference between their GFP activities, *Aequorea macrodactyla* is expected to be brighter and bluer than *Aequorea victoria.*

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