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by Dwi Haryanti

Submission date: 07-Apr-2022 08:08AM (UTC+0700)
Submission ID: 1803851084
File name: elopmental_changes_in_the_intensity_and_distribution_pattern.pdf (2.28M)
Word count: 7476
Character count: 41118

Galaxea, Journal of Coral Reef Studies 21: 13–25 (2019)

Original paper

Developmental changes in the intensity and distribution pattern of green fluorescence in coral larvae and juveniles

Dwi HARYANTI[1,*,] and Michio HIDAKA[2,*,***]**

[1] Graduate School of Engineering and Sciences, University of the Ryukyus, Nishihara, Okinawa 903−0213, Japan
[2] Department of Chemistry, Biology and Marine Science, University of the Ryukyus, Nishihara, Okinawa 903−0213, Japan

* Corresponding author: D. Haryanti, M. Hidaka
E-mail: [1] dwiharyanti@gmail.com, [2] coral35@air.ocn.ne.jp
** Present Address: Marine Science Department, Faculty of Fisheries and Marine Sciences, Diponegoro University, Jl. H. Prof. Soedarto, S.H., Tembalang, Semarang Indonesia, Tel/Fax +62 24 7474698
*** Present address: Gaja 16−8, Nishihara, Okinawa, 903−0112 Japan

Communicated by Hironobu Fukami (Biodiversity Editor)

Abstract Coral larvae and juveniles often exhibit green fluorescence due to green fluorescence proteins (GFP). Ontogenetic changes in the distribution pattern of green fluorescence in coral are not fully understood. We investigated changes in the intensity and distribution pattern of green fluorescence of the corals *Acropora tenuis*, *Pocillopora damicornis*, and *Isopora palifera* during early developmental stages. Green fluorescence distribution in larvae differed among the three species studied and changed after the larvae metamorphosed into primary polyps. Green fluorescence distributed mainly in the epidermis of *P. damicornis* and *I. palifera* larvae but in the gastrodermis in *A. tenuis* larvae. In *P. damicornis* and *A. tenuis* primary polyps, green fluorescence was present around the oral opening and in a ring structure below the tentacular ring. Green fluorescence was also present on the tips of the tentacles of *P. damicornis* and *I. palifera* primary polyps. Furthermore, green fluorescence intensity in *A. tenuis* primary polyps increased significantly after inoculation of symbionts, irrespective of the success of symbiont acquisition. In *I. palifera*, the fluorescence intensity decreased and, in some cases, disappeared after the acquisition of symbionts. Moreover, bleaching stress increased green fluorescence in *P. damicornis* juveniles. The present observations suggest that green fluorescence intensity in juvenile corals is influenced by the presence or acquisition of symbiont cells as well as by stressful conditions. This study also suggests that GFP might have different functions among coral species and at different developmental stages of the corals.

Keywords coral, GFP, fluorescent protein, symbiont, zooxanthellae

Introduction

Green fluorescent proteins (GFP) are the most common members of the fluorescent protein family in a wide variety of marine organisms, including scleractinian corals (Salih et al. 2000; Dove et al. 2001; Alieva et al. 2008; Gruber et al. 2008). In coral colonies, fluorescent proteins are localized in certain anatomical structures such as the oral disk, tentacles, tissue covering septa or

costae, and coenosarc tissue (Gruber et al. 2008). Multiple functions have been proposed for fluorescent proteins in scleractinian corals, e.g. contribution to coloration (Dove et al. 2001; Matz et al. 2006), photo-protection (Salih et al. 2000; Dove et al. 2001; Leutenegger et al. 2007a; D'Angelo et al. 2008; Roth et al. 2010), photosynthesis enhancement (Salih et al. 2000; Dove et al. 2001; Eyal et al. 2015), and protection from oxidative stress (Palmer et al. 2009). However, these proposed functions are still under debate. Kao et al. (2007) reported no correlation between fluorescent protein contents and natural coloration in *Montastraea cavernosa* (Linnaeus, 1767). Furthermore, a significant role of fluorescent proteins in photo-protection could not be detected in some corals (Gilmore et al. 2003; Mazel et al. 2003; Leutenegger et al. 2007b; Roth et al. 2013; Nakaema and Hidaka 2015a). Gilmore et al. (2003) showed that coral fluorescent proteins play only a minor role in chlorophyll excitation. In addition to the physiological functions described above, several studies have reported that coral fluorescent proteins are involved in signaling to other reef inhabitants, attracting algal-symbionts (Hollingsworth et al. 2005), producing camouflage for chlorophyll color thus avoiding herbivorous fishes (Leutenegger et al. 2007b; Haddock and Dunn 2015), and attracting prey (Haddock and Dunn 2015).

While fluorescent proteins are common in coral larvae, few studies on the ontogenetic changes in the pattern and expression of fluorescent proteins have been conducted (Hollingsworth et al. 2006; Kenkel et al. 2011; Roth et al. 2013; Nakaema and Hidaka 2015b). Green and red fluorescent protein expression has been reported to increase several days after spawning in larvae of the mushroom coral *Fungia scutaria* Lamarck, 1801 (Hollingsworth et al. 2006). Another study reported developmental changes in the distribution and intensity of GFP and cyan fluorescent protein (CFP) in *Seriatopora hystrix* Dana, 1846: GFP dominated in larvae while CFP dominated in adult colonies (Roth et al. 2013). Nakaema and Hidaka (2015b) observed that GFP fluorescence intensity was distributed throughout the polyp in *Galaxea fascicularis* (Linnaeus, 1767) primary polyps 16 days after settlement, while it became localized around the oral opening in primary polyps 23 or 51 days after settlement. Shinzato et al. (2012) found that expression of 10 candidate genes (including five GFP-genes) of fluorescent proteins in *Acropora digitifera* (Dana, 1846) differed between early developmental stages and adults.

Few studies have described fluorescent protein functions in corals at early life stages such as larvae and primary polyps. Hollingsworth et al. (2005) proposed the "beacon" hypothesis of fluorescent proteins in coral larvae, which highlights the possible function of fluorescent proteins as zooxanthellae attractants in pre-symbiotic coral larvae. Larvae of *S. hystrix* possess two types of GFPs, and the emission peak of the first one coincides with the excitation peak of the other suggesting potential biochemical role of larval GFPs in energy transfer (Roth et al. 2013). Yuyama et al. (2012) showed that fluorescence intensity and GFP gene expression changed in response to high temperature treatment in *Acropora tenuis* (Dana, 1846) primary polyps, and that the gene expression level of GFP increased after high-temperature treatment in polyps associated with clade D algae (now identified as *Durusdinium* sp.), while it decreased in polyps associated with clade A algae (now identified as *Symbiodinium* sp.). Yuyama and Higuchi (2014) found that the green fluorescence of *A. tenuis* juvenile polyps varied depending on associations with different *Symbiodinium* clades: a bright fluorescence was observed in juveniles associated with clade C algae, while such bright fluorescence was not observed in aposymbiotic juveniles or those associated with clade D algae.

The functions of fluorescent proteins in corals at early developmental stages remain to be studied. To explore the possible role of coral GFP at early developmental stages, detailed studies on ontogenetic changes in the fluorescence pattern and intensity would be useful. Therefore, we investigated changes in the fluorescence intensity and distribution pattern of green fluorescence in larvae, primary polyps (the first single polyp that was formed after settlement), and juveniles (polyp that has developed secondary polyps) in three coral species: *Acropora tenuis, Pocillopora damicornis* (Linnaeus, 1758), and *Isopora palifera* (Lamarck, 1816). We also investigated the effect of symbionts inoculation on green fluorescence intensity and distribution pattern in *A. tenuis* primary polyps, as well as the effect of bleaching stress on the green fluorescence intensity of *P. damicornis* juveniles.

Materials and methods

Collection of coral colonies and branches

Colony branches (about a quarter the size of each colony) were collected at the northern patch reef of Sesoko Island, Okinawa (26° 40′ 18.2″ N, 127° 52′ 34.2″ E) from 5 and 10 *Acropora tenuis* colonies (colony diameter ＞30 cm) in April 2011 and May 2013, respectively. Five branches from different *Isopora palifera* colonies (branch length 5−10 cm, diameter 4 cm) were collected from a shallow reef at Bise, Okinawa (26° 42′ 35.7″ N, 127° 52′ 52.8″ E) in June 2013. From this reef, five *Pocillopora damicornis* colonies (colony diameter ＞15 cm) were collected in June 2014. All corals were collected under Okinawan Prefecture permit No. 23−37 and No. 25−32 for the 2011 and 2013−2014 collection, respectively. Coral colonies and colony branches were transported to Sesoko Station and kept in an outdoor tank supplied with flowing unfiltered seawater.

Preparation of planulae and primary polyps

Acropora tenuis

Acropora tenuis colony branches were placed separately into plastic bowls every evening on expected spawning days around the full moon. Four out of ten *A. tenuis* colonies spawned gametes on July 2013. The released gametes were collected from the seawater surface using pipettes. The gametes collected from all colonies were mixed in plastic bowls (2 L) to induce fertilization at an ambient temperature of 28℃. After 2 h, the seawater was replaced with filtered seawater (0.2 µm) to remove remaining sperm. Beginning on the following day, embryos were kept in 2 L plastic bowls and the filtered seawater was changed daily. After approximately 4 days, planulae began to swim in the water column or crawl on the bottom of the bowls.

In a preliminary experiment in 2011, gametes were collected from five *A. tenuis* colonies on June 15. Larvae were obtained by mixing gametes from all colonies as describe above. The larvae were used for fluorescent photomicrographs shown in Fig. 1A and 1B, but all other experiments presented here were done using larvae collected in 2013.

To measure the GFP fluorescence intensity of *A. tenuis* soluble protein, we used extracts from embryos, planulae, and primary polyps. Approximately 100 µL of embryos or early planulae (6, 16, 27 h, and 3 days after fertilization) and 12- and 30-day-old swimming planulae were collected using pipettes and transferred into 2 mL tubes. Fifteen primary polyps (＞3 months old) were also sampled from the settlement plates and put into a 2 mL tube. All larvae and primary polyps were from the same fertilization trial in 2013. Seawater was removed from the tubes and samples were stored at −80℃ until analysis.

To extract soluble proteins, frozen samples were thawed on ice and each sample was homogenized using a plastic homogenizer pestle. Phosphate-buffered saline (1 mL; Sigma-Aldrich, St. Louis, MO, USA) was added to each tube, and the tubes were kept on ice for ＞15 min for extraction. Then, samples were centrifuged at 12,000×*g*, 4℃, for 5 min. Relative GFP fluorescence intensity was measured using a fluorescence microplate reader (GENios; Tecan, Crailsheim, Germany). A total of 200 µL of extracted sample was transferred to each well of 96-well plates (No. 165305; Nalgen Nunk International, Rochester, NY, USA) and relative fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence measurements were performed using three wells for each extract. The mean of five extraction replicates was used for each sampling time, with the exception of 12-day-old planulae and ＞3-month-old primary polyps, for which the number of extraction tubes was 3 and 1, respectively. The protein concentrations of the extracts were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) based on Bradford (1976). The absorption at 595 nm was measured using a microplate reader (Model 550; Bio-Rad). Bovine serum albumin (Bio-Rad) was used as the standard. Relative GFP fluorescence intensities were expressed as relative fluorescence units (RFU) per mg soluble protein.

Pocillopora damicornis

Planula larvae from five colonies of *P. damicornis* were collected using a planulae collector (Hidaka et al. 1997) from August 4 to 6, 2014. Colony fragments of *P. damicornis* were put in separate bowls supplied with running seawater. Released planulae were collected by sieving

overflowing seawater with a planula collector made of a plastic cup and 180 µm nylon mesh. On larvae release days, 2.5×2.5 cm ceramics settlement plates were placed at the bottom of the planulae collector. Some planulae settled during the first 3 days after release, while others remained swimming. The remaining swimming planulae from each colony were removed from the planulae collector and kept in 0.5 L bottles filled with filtered seawater (0.2 µm) after the three consecutive days of release. Settlement plates with attached primary polyps were moved into 2 L bowls filled with filtered seawater (0.2 µm) on the third day of release (August 6, 2014) with primary polyps derived from different source colonies kept in separate bowls. All collected planula larvae and primary polyps were maintained at room temperature (25–27℃) in the laboratory at Sesoko Station and then transported to the laboratory of Nishihara campus after the third day of collection. Planula larvae were then kept in 500 mL bottles (seawater volume ~300 mL, water temperature 27–28℃) and filtered seawater (0.2 µm) was changed daily. Settlement plates with settled primary polyps were placed in an aquarium equipped with a filter unit circulating aerated filtered seawater under 12 h:12 h light:dark conditions (light intensity, 30 µmol m^{-2} s^{-1}). After 3 weeks, primary polyps became juvenile colonies.

Isopora palifera

Planula larvae released by three out of five colony branches of *I. palifera* were collected using a planulae collector (Hidaka et al. 1997) in May 2013. Larvae released on three consecutive days (May 27, 28, 29) were pooled and kept in 1 or 2 L bowls filled with filtered seawater (0.2 µm) at room temperature (25–27℃) in Sesoko Station. Settlement plates were prepared by pre-conditioning ceramic plates (2.5×2.5 cm) in flowing unfiltered seawater for one month. This pre-conditioning provides natural settlement cues such as crustose coralline algae for larvae. The prepared settlement plates were put at the bottom of the bowls containing filtered seawater and larvae from different colony branches. Larvae were allowed to settle for 24 h. Settlement plates with primary polyps were then moved to 20 ml plastic disk (IWAKI, JAPAN) and kept at room temperature. The filtered seawater was renewed daily.

Fluorescence images and quantification of GFP fluorescence intensities of planulae larvae and primary polyps

Fluorescence photomicrographs of all larvae and primary polyps were obtained using a Nikon AZ-100 fluorescence microscope equipped with an excitation light source (Intensilight C-HGFI; Nikon, Tokyo, Japan) and a digital camera (Digital Sight DS-L2; Nikon). Three filters, a GFP-B filter (ex. 460–500, DM 505, BA 510–560) suitable for GFP green fluorescence images; a B-2A filter (ex. 450–490, DM 505, BA 520) suitable for both green and red fluorescence images; and a G-2A filter (ex. 510–580, DM 575, BA 580) for red fluorescence images, were used. In the case of *A. tenuis* larvae from the 2011 fertilization trial, fluorescence photomicrographs were obtained using an Olympus BX53® fluorescence microscope and DP72 digital camera (Olympus, Tokyo, Japan) (U-FBW filter, ex.460–495, DM 505, BA 510IF).

To assess the developmental changes in GFP fluorescence intensity of *A. tenuis* and *P. damicornis*, we measured GFP fluorescence intensity by image analysis of fluorescence photomicrographs. The dial controlling the excitation light intensity was set to the same position and the same image-capture settings were used for each species throughout this experiment. Mean and total green fluorescence intensity per individual were measured using ImageJ software (ver. 1.48b; National Institutes of Health, Bethesda, MD, USA). The green layer of the fluorescence images was obtained and converted to an 8-bit grey-scale image with the fluorescence intensity represented by grey pixel value ranging from 0 to 255. The contours of individual primary polyps and juveniles were selected manually or, in some cases, automatically, by selecting a threshold such that the selected area was equal to the green area in the original fluorescent image. The mean grey values of the selected areas were determined, and the total GFP fluorescence intensity per individual (in the inoculation experiment of *A. tenuis*) was calculated by multiplying the mean grey value by the area of the individual planulae larvae, primary polyps, and juveniles.

To examine the distribution of GFP fluorescence in *I. palifera* larvae, frozen sections of formaldehyde-fixed larvae (30 min in 4% formaldehyde in phosphate buffered saline) were prepared using a CM1100 cryostat (Leica

Biosystems, Germany) and observed under a Nikon AZ-100 fluorescence microscope as described above.

Inoculation of *A. tenuis* primary polyps with *Symbiodinium*

To investigate the effect of symbiont acquisition on the intensity and pattern of GFP fluorescence of *A. tenuis* primary polyps, primary polyps 37 days after settlement were inoculated with various types of cultured symbiotic algae as well as freshly isolated homologous algal symbionts. Fluorescence images of the primary polyps were obtained 2−4 days after inoculation and GFP fluorescence intensity was measured. The presence of symbiont cells in the inoculated polyps was also evaluated using a fluorescence stereomicroscope.

For inoculation experiment, one-week-old *A. tenuis* larvae derived from the 2013 fertilization trial were transported from Sesoko Station to Nishihara campus and kept in the laboratory at room temperature (25−26℃). *Acropora tenuis* larvae were induced to settle in plastic Petri dish (IWAKI, JAPAN) by exposing the larvae to 4×10^{-6} M Hym 248, a neuropeptide known to induce metamorphosis of *Acropora* larvae (Iwao et al. 2002), for 16 h on day 10 after fertilization to induce settlement. Primary polyps were k in another laboratory at room temperature (23−25℃) under a light intensity of 30 µmol $m^{-2}s^{-1}$. Freshly isolated symbionts from a parental colony or either one of six cultured strains of symbionts were introduced to *A. tenuis* primary polyps (37 days after settlement) for 4 h, following Harii et al. (2009). Since *Symbiodinium* clades are now partitioned into multiple genera (LaJeunesse et al. 2018), the genus name as well as clade of the cultured strains is shown when possible (Electronic Supplementary Material, ESM Table 1). Approximately 50 primary polyps in one Petri dish (6 cm in diameter, 4 mL seawater volume) were used for inoculation of each symbiont strain. The algal symbiont concentration was 1×10^{5} cells mL^{-1}. Homogenized *Artemia* was added to induce symbiont uptake following Kinzie and Chee (1979). After 4 h, seawater was replaced with fresh filtered seawater (0.2 µm) and the primary polyps were kept under the same temperature and light condition (23−25℃ and 30 µmol $m^{-2}s^{-1}$). Total green fluorescence intensity per individual primary polyp of *A. tenuis* before and 2−4 days after symbiont inoculation was calculated by image analysis as described above.

Green fluorescence intensity of *P. damicornis* juveniles under thermal stress

Experiment on *P. damicornis* juveniles was conducted to examine the effect of high temperature on symbiont density and mean green fluorescence intensity per mm^2 juvenile area. Each settlement plate containing 3-week-old *P. damicornis* juveniles was placed in a smaller plastic bottle (IWAKI, JAPAN) with 200 mL filtered seawater (0.2 µm) in an incubator at normal (27−28℃) or high temperature (32℃) for 2 w s. Light conditions inside the incubator were 30 µmol $m^{-2}s^{-1}$ with a 12 h:12 h light:dark cycle. Filtered seawater in each bottle was renewed daily. Mean green fluorescence intensities of juveniles before and after stress exposure were measured by image analysis as described above. Symbiont densities of juveniles were not quantified in this experiment, although color photomicrographs of juveniles were taken.

Statistical analysis

Differences in green fluorescence intensity among initial or control *A. tenuis* polyps and those inoculated with various types of symbiont were tested using Kruskal-Wallis and multiple comparison p-value test. GFP fluorescence intensity was compared among three *P. damicornis* developmental stages (1−3-day planulae, 1−3-day primary polyps, and 20−22-day juveniles) using ANOVA and Tukey's multiple comparison test or Kruskal-Wallis and multiple comparison p-value tests when the data did not meet the requirements for parametric analysis. Similarly, mean green fluorescence intensities were compared between juveniles before and after stress experiment using Kruskal-Wallis and multiple comparison p-value test. All statistical analyses were performed using STATISTICA software (ver. 6.0; StatSoft, Tulsa, OK, USA).

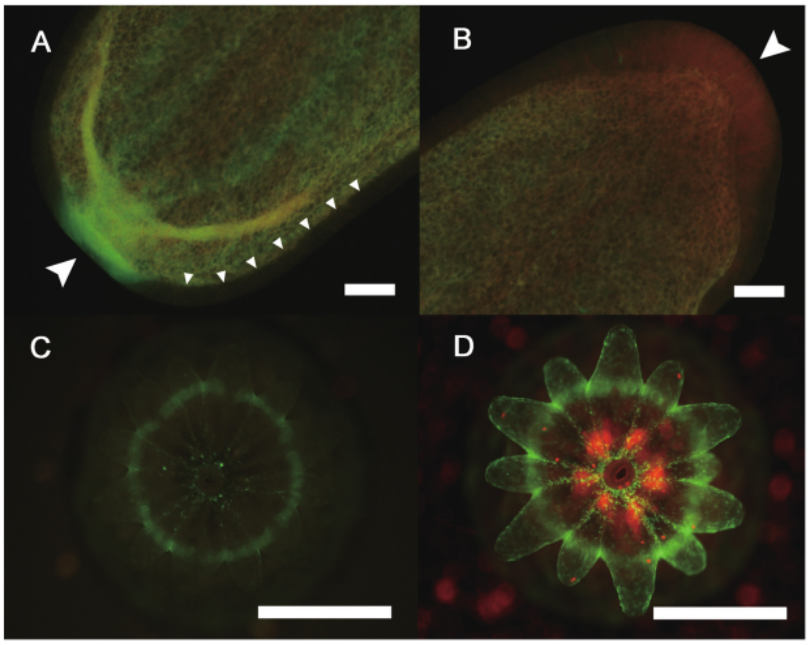

Fig. 1 Pattern of green fluorescence in the planula and primary polyps of *Acropora tenuis*. **A**, Oral part of a planula (12 days after fertilization). Green fluorescence was distributed in the gastrodermis and oral area (arrowhead). The epidermis (shown by small arrowheads) lacked green fluorescence. **B**, Aboral part of the same planula shown in **A**. The gastrodermis exhibited green fluorescence while the epidermis (arrowhead) of the aboral part exhibited red fluorescence (RFP). **C**, Primary polyp 11 days after settlement (52 days after fertilization). Green fluorescence was concentrated in an O-ring like structure surrounding the base of the tentacular ring. **D**, Primary polyp 3 days after inoculation of cultured *Symbiodinium* cells (clade B, *Breviolum* sp.). Red fluorescent particles in the polyp indicate *Symbiodinium* cells. Fluorescence images **A** and **B** were obtained with the same acquisition settings using an Olympus BX53® fluorescence microscope and DP72 digital camera (U-FBW filter, ex.460−495, DM 505, BA 510IF). Fluorescence images **C** and **D** were obtained using the same image acquisition settings, using Nikon AZ-100 fluorescence microscope with a B-2A filter (Ex 450−490/Em 520, DM 505, BA 520), exposure time/camera gain＝1 s/170. Scale bars＝100 µm (**A** and **B**) and 500 µm (**C** and **D**).

Results

Developmental changes in green fluorescence intensity and distribution pattern

In *A. tenuis*, no clear green fluorescence was observed in early embryos or early planulae. Bright green fluorescence was observed in the gastrodermis of swimming planulae (12 days after fertilization), especially in the oral part (Fig. 1A). Red fluorescence was also observed in the epidermis of the aboral area (Fig. 1B). After settlement and metamorphosis of planula larvae into primary polyps, green fluorescence became concentrated to an O-ring like structure at the base of the tentacular ring (Fig. 1C and 1D).

Green fluorescence was not detected in the extract of *A. tenuis* early embryos or the early planula stage (3 days after fertilization). When the larvae started to swim actively (12 and 30 days after fertilization), green fluorescence (relative fluorescence unit/mg soluble protein) of *A. tenuis* larvae extract increased (Fig. 2). Green fluorescence tended to decrease in primary polyps older than 3 months, although the difference was not tested statistically because the number of biological replicates was small (Fig. 2).

In *P. damicornis*, green fluorescence was distributed in

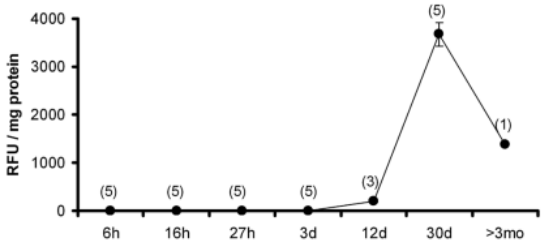

Fig. 2 Changes in the green fluorescence intensity of *Acropora tenuis* during early development. Green fluorescence intensity is expressed as relative fluorescent units (RFU) per mg soluble protein. Numbers in parentheses indicate the number of extraction procedures repeated using the same batch of larvae or primary polyps from the same fertilization trial. Mean±SD is shown except for the >3 mo old primary polyps. SD bars less than the size of the symbols are not shown.

the epidermis of planulae, while symbiont cells (red chlorophyll fluorescence) were distributed in the gastrodermis of planulae (Fig. 3A) and newly settled primary polyps (Fig. 3B). Green fluorescence intensity was high in the oral and aboral parts of the planula. After settlement, green fluorescence was distributed throughout the polyp but was concentrated especially around the oral opening. In 6−8-day-old primary polyps or juveniles 20−22 days after settlement, green fluorescence was distributed especially in the oral opening, tentacle tips, and O-ring like structure at the base of the tentacular ring (Fig. 3C and 3D).

Mean green fluorescence intensity varied among samples derived from different colonies, in planulae ($p<0.05$, ANOVA), 1−3-day-old primary polyps ($p<0.01$, ANOVA), and 20−22-day-old primary polyps ($p<0.01$, Kruskal-Wallis test) (Fig. 3E). The mean green fluorescence intensity decreased and was localized in certain areas as the primary polyps grew from 1−3 to 20−22 days (Tukey's multiple comparison test, $n=5$, $p<0.05$) (Fig. 3E).

In swimming larvae of *I. palifera*, green fluorescence was distributed evenly in the epidermis, but no green fluorescence was observed in the aboral area (Fig. 4A and 4B). After settlement, green fluorescence was distributed throughout the primary polyp, but high green fluorescence intensity was observed in the tentacles and oral region (Fig. 4C). Green fluorescence was concentrated in the tentacle tips after the primary polyps developed into juveniles and acquired symbionts (Fig. 4D). In some juveniles, green fluorescence was not detected (photos not shown).

Effects of symbiont inoculation on green fluorescence pattern of *A. tenuis* primary polyps

Changes in green fluorescence intensity of *A. tenuis* primary polyps after inoculation trial with different strains of symbionts were examined using fluorescence micrographs. When primary polyps of *A. tenuis* were inoculated with either one of six cultured symbiont strains or symbiont freshly isolated from a parental colony, primary polyps took up many, a few, or no symbiont cells depending on the type of symbiont (Electronic Supplementary Material, ESM Fig. 1). Many symbiont cells were observed in the primary polyps of *A. tenuis* when strains Y106 (*Symbiodinium* sp.), KB8 (*Symbiodinium* sp.), or K100 (*Breviolum* sp.) were inoculated. A few symbiont cells appeared in 10−15 of 25 primary polyps 2−3 days after inoculation when cells of *Symbiodinium* strain K111 (*Durusdinium* sp.) or those freshly isolated from parental colony (*Cladocopium* sp., previously mentioned as subclade C3) were inoculated. Most of the primary polyps (24 out of 25 primary polyps) inoculated with strain Y103 (*Cladocopium* sp.) and K102 (*Fugacium* sp.) failed to take up symbiont cells. Green fluorescence intensity increased after inoculation in all cases, even when symbiont acquisition was unsuccessful (Fig. 5). After inoculation with any of the six strains or freshly isolated symbiont cells, green fluorescence intensity of primary polyps was significantly higher than the initial green fluorescence intensity ($p<0.05$, multiple comparison p-value test). Similarly, all inoculated primary polyps showed significantly higher green fluorescence intensity than the control primary polyps (without inoculation) ($p<0.05$, multiple comparison p-value test), except those inoculated with strain Y103 (*Cladocopium* sp.).

Mean GFP fluorescence intensity and symbiont density in juveniles of *Pocillopora damicornis* under thermal stress

After 2 weeks of exposure to normal (27−28℃) or high

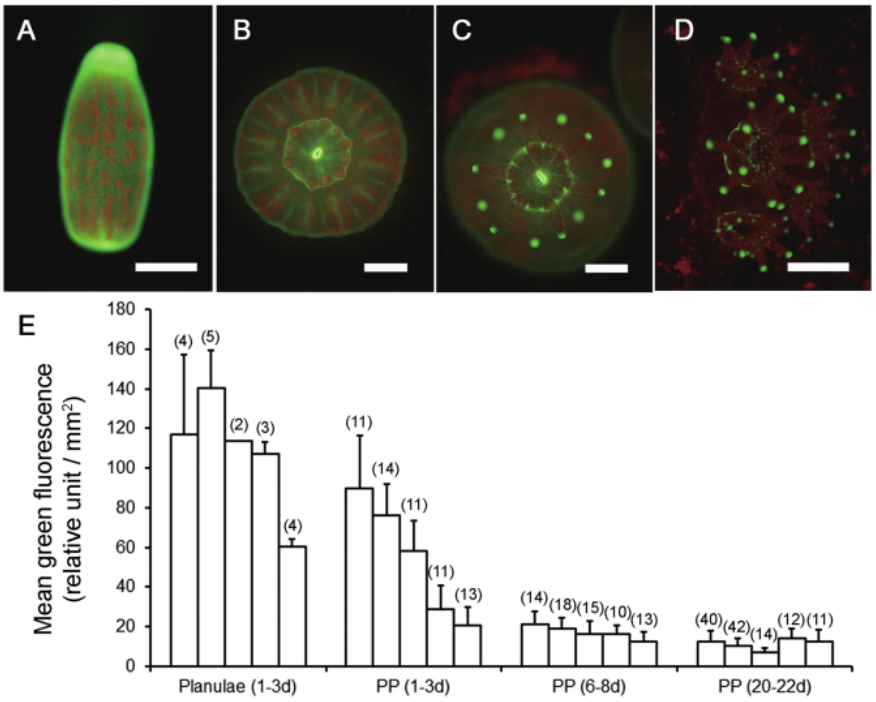

Fig. 3 Developmental changes in the pattern and intensity of green fluorescence in *Pocillopora damicornis*. **A**, Planula (1−3 day old). Green fluorescence was distributed in the epidermis, while algal symbiont cells (red chlorophyll fluorescence) were distributed in the gastrodermis of the planula. Green fluorescence intensity was high in the oral and aboral parts of the planula. **B**, Newly settled primary polyps (1−3 days after settlement). Green fluorescence was distributed throughout the polyp but was concentrated around the oral opening. **C**, Primary polyp (6−8 days after settlement). Green fluorescence was distributed around the oral opening, in the tentacle tips, and the O-ring like structure at the base of the tentacular ring. **D**, Juvenile (20−22 days after settlement). Green fluorescence was localized around the oral opening, in the tentacle tips, and the O-ring like structure in each polyp. **E**, Mean Green fluorescence intensity of a planula or a primary polyp. Each bar indicates Mean±SD for planulae or primary polyps from the same source colony. Numbers in parentheses indicate the number of planulae or primary polyps measured. B-2A filter (Ex 450−490/Em 520, DM 505, BA 520), exposure time/camera gain＝1/3s/140. Scale bars＝500 μm.

temperature (32℃) in a 200 mL plastic bottle, the primary polyps became pale compared to those before the stress treatment (Fig. 6A−C). Green fluorescence intensity was considerably higher after 2-week stress treatment, and green fluorescence distribution extended to the entire surface area of juveniles, including coenosarcs, where green fluorescence was absent or very low before stress treatment (Fig. 6D−F). Mean green fluorescence intensity was significantly higher in juveniles kept in a small bottle at both temperatures than those kept in a large (10 L) control tank before stress treatment (Fig. 6G) (Multiple comparison p-value test, $p < 0.01$).

Discussion

Changes in the intensity and distribution pattern of green fluorescence during early development

Fluorescence measurements of soluble proteins extracted from *A. tenuis* at early developmental stages (four-cell stage to 3-day-old planula) showed no green fluorescence, and this was first detected in swimming larvae (12-day-old planulae) (Fig. 2). Similarly, Hollingsworth et al. (2006) reported that green and red fluorescence proteins expression increased several days after fertili-

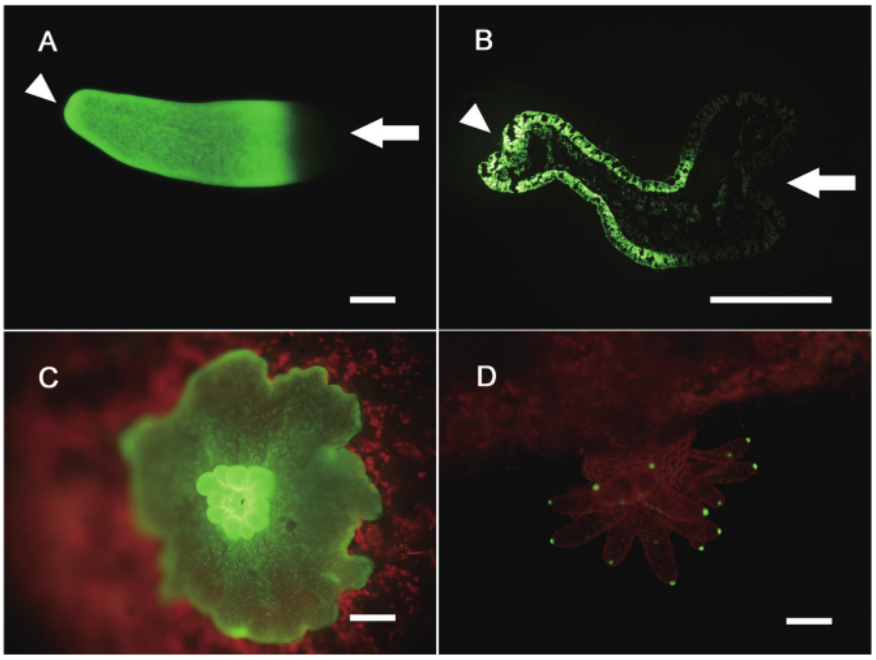

Fig. 4 Developmental changes in green fluorescence distribution in *Isopora palifera*. **A**, A planula 1 day after release. Green fluorescence was distributed in the epidermis of the planula, with the exception of the aboral area. **B**, Cryosection of a planula. Green fluorescence is localized in the epidermis of the oral part. **C**, Primary polyp (11-day old, 3 days after settlement). Green fluorescence was distributed throughout the primary polyp, with strong green fluorescence in the tentacles and oral region. **D**, Juveniles after symbiont acquisition (76-day old). GFP was distributed only in the tentacle tips. Arrowhead: oral area of planula; Arrow: aboral area of planula. B-2A filter (Ex 450-490/Em 520, DM 505, BA 520), exposure time/camera gain＝1/3 s/140. Scale bar ＝500 μm.

zation in non-symbiotic larvae of *Fungia scutaria*. It is likely that GFP expression starts at planula stage in these corals.

The distribution patterns of green fluorescence in larvae were different among the three species studied. Larvae of *P. damicornis* exhibited green fluorescence in the epidermis, and *A. tenuis* larvae in the gastrodermis. Larvae of *I. palifera* exhibited green fluorescence in the epidermis of the whole body, except the aboral end.

Green fluorescence distribution pattern changed after larvae metamorphosed into primary polyps. *Pocillopora damicornis* and *A. tenuis* primary polyps showed an O-ring pattern of green fluorescence at the base of the tentacular ring. In *P. damicornis* primary polyps, green fluorescence intensity was markedly high around the oral opening. GFP fluorescence has been observed in the area surrounding the mouth during development of primary polyps of *Galaxea fascicularis* (Nakaema and Hidaka 2015b). Fujiwara et al. (2012) reported that green fluorescence in *A. tenuis* live planulae revealed typical bipolar neurons with varicosities along axons. Contrastingly, *P. damicornis* polyps showed nerve rings around the mouth (cited in Koizumi et al. (2015) as unpublished observation). It is interesting whether the O-ring like pattern of green fluorescence corresponds to the nerve ring.

Green fluorescence was also present at the tentacle tips of *P. damicornis* and *I. palifera* primary polyps. The different green fluorescence distribution pattern among species and between developmental stages suggests that the function of GFP might be different among species as well as between developmental stages.

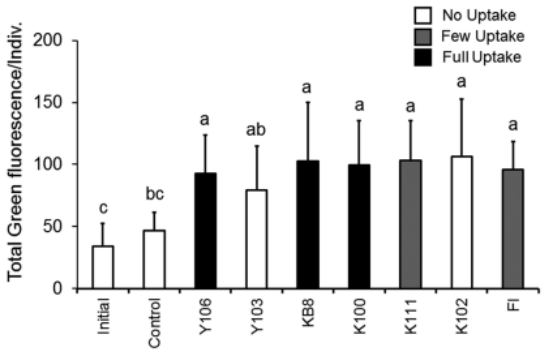

Fig. 5 Green fluorescence intensities of *Acropora tenuis* primary polyps before and after inoculation with various types of symbiont. Black and white bars indicate primary polyps with and without symbiont uptake, respectively. Grey bars indicate that some polyps (10–13 out of 25 polyps) took up small numbers of symbiont cells. Bars marked with the same letters are not significantly different from one another ($p < 0.05$, Multiple comparison p-value test). Mean±SD (n=80 for initial, n=25 for each strain and the control).

Possible function of GFP: symbiont attraction

Green fluorescence was observed in the gastrodermis and around the oral opening of *A. tenuis* larvae (Fig. 1A). After settlement and metamorphosis of planula larvae into primary polyps, green fluorescence was concentrated to an O-ring like structure at the base of the tentacular ring (Fig. 1D).

GFP fluorescence intensity in *A. tenuis* primary polyps increased after inoculation of symbionts regardless of whether symbiont uptake occurred or not (Fig. 1C and 1D, and Fig. 5). This study indicates that the presence of symbiotic algae in the surrounding seawater influenced the green fluorescence intensity in *A. tenuis* primary polyps. Hollingsworth et al. (2005) suggested that GFP might attract algal symbionts since *Symbiodinium* exhibits positive phototaxis to green light. In the present study, the fluorescence intensity was measured 2–4 days after inoculation. Therefore, it is not clear whether the increased green fluorescence was a response to some stress condition caused by the inoculation process or related to the possible function of GFP in attracting symbionts. Yuyama and Higuchi (2014) observed bright green fluorescence in *A. tenuis* juveniles associated with clade C1 algae but little fluorescence in those associated with clade D algae and suggested that corals experience oxidative stress when they initially acquire clade C1 algae. It remains to be studied whether GFP in *A. tenuis* primary polyps is involved in acquisition of symbionts from the environment. Green fluorescence was high in *I. palifera* larvae and newly settled primary polyps, but it decreased or disappeared in juveniles that had acquired symbiotic algae. Future studies should focus on whether GFP fluorescence of juveniles increases when symbiotic algae are present around them and decreases once stable symbiosis is established in these corals.

Possible function of GFP: photoprotection, camouflage, and prey capture

In *P. damicornis* larvae, green fluorescence was distributed in the epidermis, covering algal symbiont in the gastrodermal cells (Fig. 3A). GFP was suggested to have photoprotective function for symbionts (Salih et al. 2000; Roth et al. 2010). GFP might also function as camouflage to protect coral from herbivorous fishes (Leutenegger et al. 2007b; Haddock and Dunn 2015). The presence of green fluorescence in the epidermis of *P. damicornis* larvae suggests that GFP has a photoprotective function and/or functions as screen for chlorophyll fluorescence facilitating avoidance of herbivorous predators. These possibilities should be tested in a future study. Experiments with coral larvae whose GFP gene expression is modified might be fruitful.

Green fluorescence intensity in *P. damicornis* larvae decreased following their metamorphosis and settlement on the substrate (Fig. 3E). In 1–3-day-old primary polyps, green fluorescence was distributed throughout the polyp and concentrated especially around the oral opening (Fig. 3B). In 6–8-day-old primary polyps, green fluorescence was distributed almost exclusively in the oral opening, tentacle tips, and O-ring like structure at the base of the tentacular ring (Fig. 3C). Similarly, when *I. palifera* primary polyps developed into juveniles and acquired symbionts, green fluorescence became concentrated in the tentacle tips (Fig. 4D). However, green fluorescence was not detected in some juveniles. GFP functions as a prey attractant in various reef animals (Haddock and Dunn 2015). Gruber et al. (2008) also suggested that targeted expression of fluorescent proteins may function as a

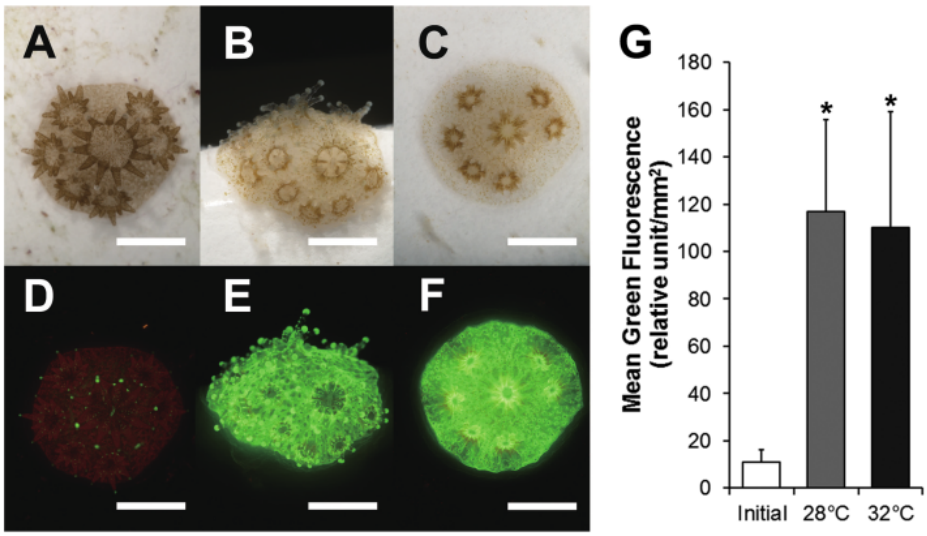

Fig. 6 Effect of bleaching stress on the green fluorescence intensity of juveniles of *Pocillopora damicornis*. Bright field and fluorescence images of 3-weeks-old juveniles before stress treatment (**A** and **D**), after 2-week treatment in a small bottle at 28℃ (**B** and **E**) or at 32℃ (**C** and **F**). **G**, The mean green fluorescence intensity of juveniles before and after 2-week stress treatment. Mean±SD (n＝119 for initial, n＝39 and 36 for 28℃ and 32℃ treatment, respectively). *, significant increases in the fluorescence intensity when compared with those before treatment (Multiple comparison p-value test, $p<0.01$). Scale bar＝1 mm

signaling mechanism to organisms such as copepods, with sensitivities in the range of green and red fluorescence. Since the tentacle tips (acrospheres) deploy many nematocysts and *P. damicornis* is known to capture zooplankton as prey (Clayton and Lasker 1982), it is likely that GFP fluorescence in the tentacle tips of *P. damicornis* juveniles might function to attract prey (Haddock and Dunn, 2015). The idea that the GFP fluorescence at tentacle tips of *P. damicornis* and *I. palifera* juveniles might facilitate capture of prey should be tested in a future study.

Effects of environmental stress on green fluorescence

After 2-week treatment in a small (200 mL) bottle, *P. damicornis* juveniles became pale at both normal (27–28℃) and high (32℃) temperatures, probably because the small volume of seawater induced bleaching stress. At the same time, mean green fluorescence intensity increased and the green fluorescence, which had been localized to tentacle tips, around the mouth, and O-ring like structure, extended to the entire surface area of the juvenile (Fig. 6D–F). The present observation shows that, under bleaching stress, GFP fluorescence not only increased but also appeared in the coenosarc, where green fluorescence was weak or not observed before. Roth and Deheyn (2013) showed that heat-treated *Acropora yongei* expressed strong fluorescence despite a reduced GFP concentration, and they suggested that a reduction in algal symbiont density led to decreased absorbance of green light emitted by GFP. However, the present study suggests that GFP expression in *P. damicornis* juveniles increased in response to bleaching stress. Recently, Aihara et al. (2019) demonstrated that algal symbionts were attracted to green fluorescence emitted by both endogenous GFP of live coral fragments and an artificial green fluorescence dye. They suggested that green fluorescence may help corals acquire symbiotic algae from the environment at early life stage as well as during recovery from bleaching. Since GFP is considered to have photoprotective and antioxidant functions, increased expression of GFP might also facilitate protection of remaining or newly acquired algal symbionts by the coral host.

Conclusions

The distribution pattern of green fluorescence in *Acropora*, *Isopora*, and *Pocillopora* larvae differed among genera. As the larvae metamorphosed to primary polyps, green fluorescence became more or less concentrated in specific anatomical structures, such as tentacle tips, the oral area, or the O-ring at the base of the tentacular ring. The developmental changes in GFP distributions were also different among species. The present study suggests GFP might have different functions among coral species as well as between developmental stages. GFP fluorescence intensity increased after symbiont inoculation in *A. tenuis* primary polyps and decreased in *I. palifera* juveniles which had acquired symbiotic algae. In *P. damicornis* juveniles under bleaching stress, green fluorescence increased and extended to the whole body. This study indicates the need for further exploration of GFP function in corals at early development stages, focusing on the physiological and ecological roles of coral fluorescent proteins.

Acknowledgements

We would like to thank Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, where part of this study was conducted. This study was supported in part by the International Research Hub Project for Climate Change and Coral Reef/Island Dynamics of University of the Ryukyus, and by Center for Strategic Research Project, University of the Ryukyus. We would like to thank anonymous reviewers for helpful comments, which improved the manuscript.

Compliance

All corals used in this study were collected under Okinawan Prefecture permit No. 23−37 and No. 25−32 for the 2011 and 2013-2014 collection, respectively.

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∗ESM Table 1 and ESM Fig. 1 can be downloaded from the J-STAGE website:
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Received: 11 December 2018
Accepted: 6 August 2019

Developmental changes in the intensity and distribution pattern of green fluorescence in coral larvae and juveniles

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