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GFP distribution and fluorescence intensity in *Galaxea fascicularis*: developmental changes and maternal effects

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Abstract

Scleractinian corals exhibit various colors depending on the host fluorescent proteins and non-fluorescent chromoproteins. It is not understood whether the color patterns are determined genetically or influenced by the environment. We performed breeding experiments between green (Gs) and brown (B) color morphs of *Galaxea fascicularis*, wherein Gs morphotypes have much higher green fluorescent protein (GFP) content than that of B morphotypes. Larvae that developed from B morphotype eggs showed significantly higher average GFP fluorescence intensity per polyp area than those from Gs morphotype eggs, regardless of paternal morphotypes. Similarly, juveniles derived from B × B crosses showed higher fluorescence intensity than those derived from Gs × Gs crosses. The GFP pattern changed after settlement in that it increased in concentration around the mouth. No difference was observed among crosses. This is the first study to show a possible maternal effect on GFP intensities in coral larvae and juveniles.

Key words: Color morphotypes, Development, Fluorescent protein, GFP, *Galaxea fascicularis*

Introduction

Scleractinian corals exhibit various colors depending on the host pigments, which consist of fluorescent proteins (FPs) and non-fluorescent chromoproteins. Some studies have reported that FP expression in corals changes with environmental stress or light conditions. For example, FP

expression is down-regulated by heat stress (Smith-Keune and Dove, 2007; Desalvo et al., 2008; Rodriguez-Lanetty et al., 2009; Roth and Deheyn, 2013) or cold stress (Roth and Deheyn, 2013) but up-regulated by increased light intensity (Roth et al., 2010) or blue light (D'Angelo et al., 2008). However, various color morphotypes (hereafter 'morphs') of the same coral species have been observed side by side under the same environment (Takabayashi and Hoegh-Guldberg, 1995; Salih et al., 2000; Nakaema and Hidaka, 2015). Shinzato et al. (2012) reported that in *Acropora digitifera* FP expression changes during development. In juveniles of *A. tenuis*, the symbiont clade influences GFP expression and response to high temperature stress (Yuyama et al., 2012). However, it is not well understood how the color patterns of corals change during early developmental stages and whether they are determined genetically or influenced by physiological responses to the environment.

The reef-building coral *Galaxea fascicularis* (Linnaeus, 1767) has several color morphs that show different distribution patterns. The Gs morph is green around the oral disc and tentacles, while the entire polyp of the B morph is brown (Hidaka and Yamazato, 1985). They live side by side in shallow reefs in Okinawa but contain different amounts (green fluorescence intensity per unit soluble protein) of GFP (Nakaema and Hidaka, 2015).

The objectives of this study were to understand how the intensity and distribution pattern of FPs change during early developmental stages, how the parent color pattern affects FP expression, and how the presence and type of symbiont affect the color pattern of juveniles.

Materials and Methods

Sample collection and larvae preparation

Colonies of Gs and B morphs of *G. fascicularis* were collected from depths of 0.5–1 m during low tide at Zampa and Sesoko reefs, Okinawa Island, Japan in June and July 2013. The colonies were maintained in an outdoor tank supplied with running unfiltered seawater at Sesoko Station, University of the Ryukyus, Okinawa until spawning in July 2013. The tank was shaded by a mesh screen and the light levels reached 700-800 μmol m^{-2} s^{-1} at noon. All individual colonies were placed separately in containers with 2–3 L unfiltered seawater before the expected time of spawning (7–11 p.m., 6–10 days after the full moon in July 2013). When collected colonies released egg bundles or pseudo-egg/sperm bundles, we collected them with a pipette and transferred them to 50-ml falcon tubes containing filtered seawater (0.22 μm, FSW). Seawater containing sperm was taken from the tube containing pseudo-egg/sperm bundles, and sperm and eggs were mixed in

additional containers (1 L) with FSW. A small volume of seawater collected from the containers with parental colonies was added to the containers to increase the fertilization rate (Abe et al., 2008).

We prepared four cross combinations of the two color morphs: sperm × eggs, Gs × Gs, Gs × B, B × Gs, and B × B. Each consisted of four crosses with eggs from four female colonies and sperm from one male colony. A total of 16 crosses were prepared from eight female (four Gs and four B morph colonies) and two male colonies (one Gs and one B morph colonies). However, a total of 14 crosses were evaluated as we were unable to obtain fertilized eggs in two B × Gs crosses.

After fertilization, we kept larvae in 1–2-L containers with FSW, which was changed daily. Seven days after fertilization, three to six actively motile larvae were selected randomly from each cross and mounted in a seawater drop on a glass slide. Fluorescence photomicrographs of the larvae were taken under a fluorescent stereoscope AZ100 (Nikon) equipped with GFP-B (excitation (ex) 460–500 nm, emission (em) 510–560 nm for GFP)/ G-2A (ex 510–560 nm, em 590 nm for red fluorescent protein (RFP) and chlorophyll fluorescence) filters and a digital camera (Digital Sight DS-Fi1, Nikon, Tokyo, Japan). Although the spectrum of GFP of *G. fascicularis* indicated that the optimum excitation and emission wavelength were 507 and 515 nm, respectively, we used an available GFP-B filter. Images of larvae were taken under the same settings; an exposure time of 1 sec and a gain value 100. Pixel resolution of fluorescence intensity was 8-bit, scaling from 0–255.

Settlement and *Symbiodinium* inoculation

Eight days after fertilization, 50–100 larvae from each cross were placed in two 5-cm Petri dishes with FSW. We replaced the FSW daily until most larvae settled on the bottom of the dishes. After 10 days, unsettled larvae were removed. The primary polyps were maintained for 23 days further until the inoculation experiment.

For inoculation of the primary polyps, *Symbiodinium* were isolated from one Gs and one B morph colony that had been used as sperm donors. The clade type of the *Symbiodinium* was determined by PCR-RFLP analysis using the restriction enzyme *Taq*I. The *Symbiodinium* associated with eight female colonies (4 Gs and 4 B morph colonies) belonged to clade D, while one Gs male colony and one B male colony used as sperm-donor were associated with *Symbiodinium* of clade C and D, respectively. Isolated *Symbiodinium* (1.0×10^5 cells $\cdot$ ml^{-1}) of either clade C or D were introduced to primary polyps. After an inoculation period of 4 h, polyps were rinsed in FSW and maintained for 30 days, with FSW changed daily. Fluorescence photographs

of the juveniles were taken as described above except a longer exposure time (2 sec) before and 2, 14, and 28 days after *Symbiodinium* inoculation.

Image analysis

The GFP fluorescence intensity was measured on the green channel of fluorescence photomicrographs using ImageJ (Research Services Branch, National Institutes of Health, Bethesda, MD, USA). We traced the outline of larvae or juveniles, and the average intensity within that area was measured. The Integrated Density (mean gray value multiplied by the area) of chlorophyll fluorescence was measured as the amount of chlorophyll fluorescence from the red channel of the fluorescence photomicrographs. However, in the case of chlorophyll fluorescence, the area surrounding the mouth was removed from the analyses as it contained RFP (see Fig. 2B). In apo-symbiotic polyps (polyps just before inoculation or those that failed to take up *Symbiodinium* cells), RFP fluorescence was limited to the area surrounding the mouth. Thus, we considered that red fluorescence in the polyp area other than the peri-oral area was due to chlorophyll fluorescence of *Symbiodinium* cells. In this study, the amount of chlorophyll fluorescence was used as an indicator of *Symbiodinium* abundance.

Statistical analyses

Statistical analyses were conducted using R v. 3.0.1 (R core development team). Equality of variances was assessed using an *F*-test and normality by a Shapiro-Wilk normality test. Welch's t-test was used when the equality of variances was rejected. The GFP fluorescence intensities of larvae derived from Gs morph eggs and those derived from B morph eggs were compared using Student's t-test. Fluorescence intensities of three to six larvae were measured in each cross and the mean value was used as statistical unit for the statistical analysis, except for one cross of Gs × Gs combination, where we obtained fluorescence intensity from only one larva. Differences in GFP fluorescence intensities among juveniles of different cross combinations were tested using Student's t-test. When the number of replicates was less than three, we excluded the data from the statistical analyses.

Results

Green fluorescence was observed mainly in the ectodermal layer of the larvae, and red fluorescence was more or less evenly distributed (Fig. 1A). Similar GFP and RFP distributions were observed in larvae from all breeding combinations. However, the GFP fluorescence intensity in larvae was affected by the color morph of the maternal colonies. When a Gs morph colony was used as the sperm donor, larvae derived from B morph eggs showed significantly higher GFP fluorescence intensity than those derived from Gs morph

eggs (t-test, $P < 0.05$, Fig. 1B). When a B morph colony was used as sperm donor, the same tendency was observed with larvae derived from B morph eggs: higher GFP intensities than those derived from Gs morph eggs, although a statistical analysis was not performed due to a low number of replicates (n = 2) in the B × Gs crosses. Additionally, we could not examine the influence of sperm donor's color morph as only one sperm donor colony was used in this study.

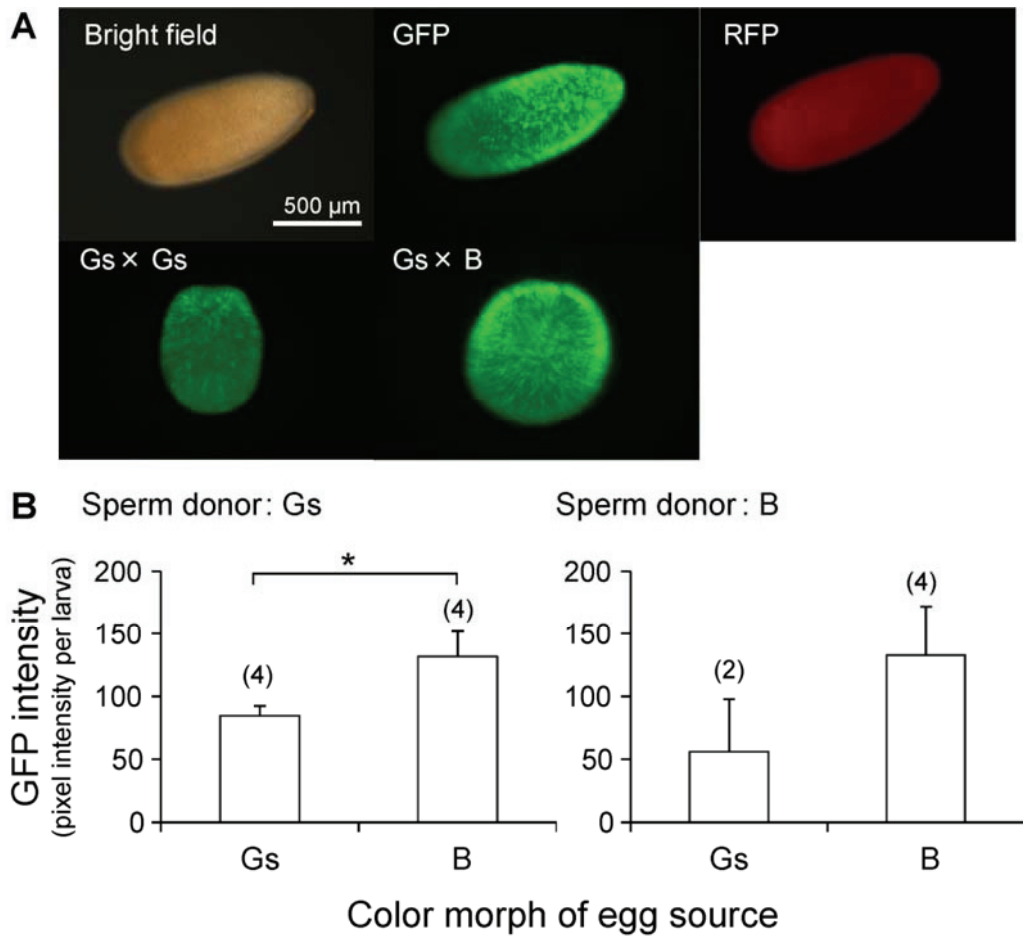

Fig. 1. Distribution and fluorescence intensity of GFP and RFP in larvae of *G. fascicularis*. **A**, Bright field micrograph and fluorescence micrographs showing GFP and RFP images of a larva derived from a B morph parent (upper photos). Fluorescence micrographs of larvae derived from different crosses (sperm × eggs: Gs × Gs, Gs × B) (lower photos). **B**, Fluorescence intensities of larvae derived from different crosses. The asterisk indicates a significant difference (t-test, $P < 0.05$). Mean ± SD. The numbers in the bracket indicate the number of crosses.

The fluorescence distribution of juveniles changed during development. In primary polyps 16 days after settlement, GFP, which appeared as green dots or granules, extended throughout the polyps (Fig. 2A). As the juveniles developed further (23 and 51 days after settlement), fluorescence faded, only remaining in the area surrounding the mouth (Fig. 2B, C). RFP distribution pattern also showed similar changes as described above.

The GFP intensity of juveniles from the B × B combination was significantly higher than that of polyps from the Gs × Gs combination on day 44 and/or 51 (t-test, $P < 0.05$) (Fig. 3). As the number of live Gs × Gs polyps inoculated with clade D *Symbiodinium* decreased to two on day 51, we did not perform a statistical analysis for Gs × Gs polyps on day 51.

The GFP intensity in juveniles from the B × B combination tended to increase with time while those from Gs × Gs combination remained unchanged (Fig. 3). Juveniles from the Gs × B combination exhibited intermediate patterns between Gs × Gs and B × B combinations.

In juveniles inoculated with clade D *Symbiodinium*, the amount of chlorophyll fluorescence tended to increase after inoculation. However, in juveniles inoculated with clade C *Symbiodinium*, the amount of chlorophyll fluorescence appeared unchanged after inoculation for both Gs × Gs and B × B combinations (Fig. 3).

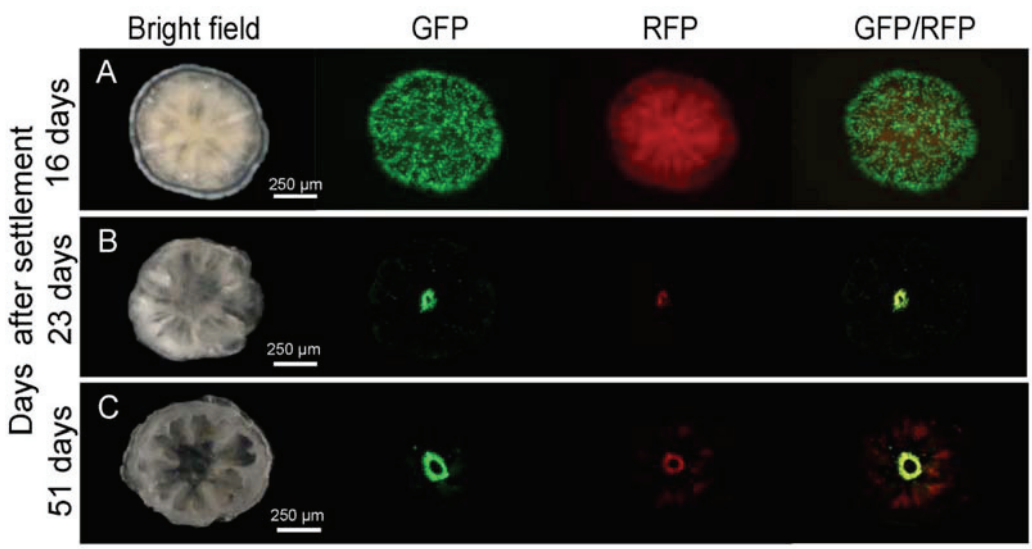

Fig. 2. Distribution of GFP and RFP in juveniles of *G. fascicularis*. Bright field and fluorescent micrographs were taken 16 (**A**), 23 (**B**), and 51 (**C**) days after settlement (7 days before, immediately before, and 28 days after inoculation) in the combination Gs × B. Epifluorescence photomicrographs were taken using the following filters: GFP-B for GFP, G-2A for RFP, and B-2A for GFP/RFP.

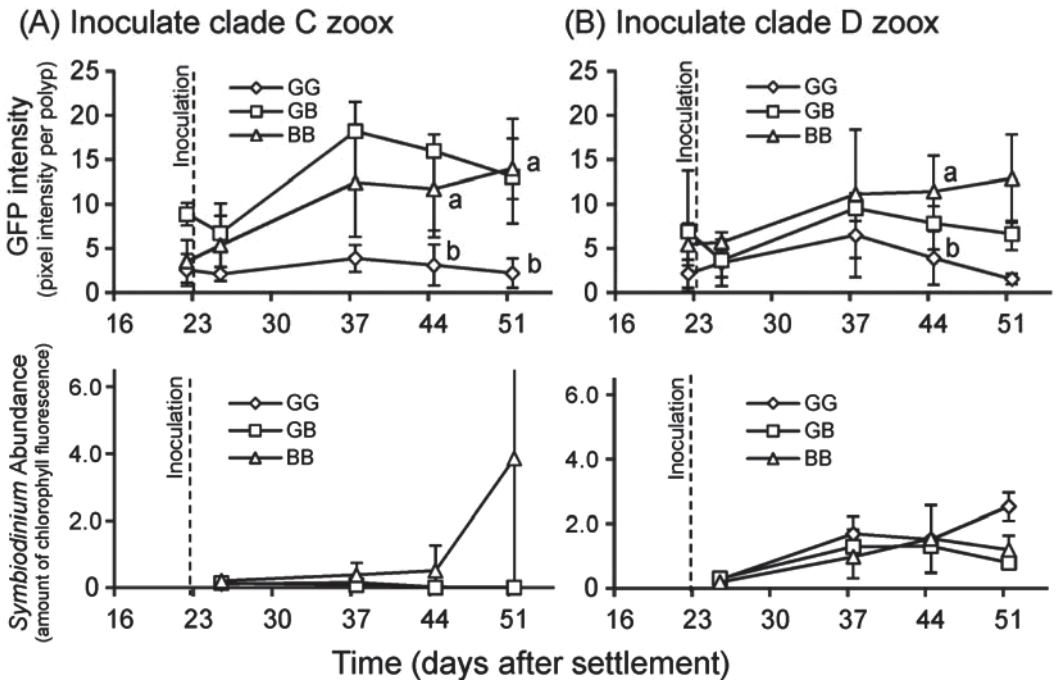

Fig. 3. Changes in the fluorescence intensities of GFP and *Symbiodinium* abundance in juveniles inoculated with clades C or D *Symbiodinium*. The fluorescence intensities of polyps derived from different crosses (sperm × eggs: Gs × Gs, Gs × B, and B × B) on days 22, 25, 37, 44, and 51 are shown. Different letters indicate significant differences in GFP intensity between the combinations Gs × Gs and B × B on day 44 or 51 (t-test, $P < 0.05$). Mean ± SD; n = 3–4 crosses, except Gs × B juveniles inoculated with clades C or D (n = 2) and Gs × Gs juveniles inoculated with clade D on day 51 (n = 2).

Discussion

GFP was distributed throughout the bodies of *G. fascicularis* larvae (Fig. 1). GFP was also distributed as dots or granules in juveniles, although it became concentrated around the mouth in later stages (Fig. 2). Thus GFP distribution changed with development, and similar developmental changes were observed in all breeding combinations.

Larvae that developed from B morph eggs showed significantly higher GFP fluorescence intensity than those from Gs morph eggs, regardless of paternal color morphs. This indicates that GFP expression in larvae is affected by maternal colonies. Similarly, GFP fluorescence intensity was higher in those juveniles that developed from the B × B combination than those from the Gs × Gs

combination during later stages of development (44 or 51 days after settlement). Additionally, GFP fluorescence intensity in juveniles tended to increase with time in the B × B combination, while it remained at low levels in the Gs × Gs combinations.

In this study, Gs and B morph colonies were collected from similar depths (0.5–1 m) during low tide. Colonies of both morphs were often found side by side in the same environment. Therefore, it is likely that the colonies of the two color morphs are different genetically. However, it is not clear whether the maternal effect on the GFP intensity of larvae and juveniles observed in this study was caused by the genotype of the offspring or by maternal factors such as mRNA or proteins. It is interesting that the GFP intensity in larvae or juveniles was higher when they developed from eggs from B morph colonies, which exhibit much lower GFP intensity than that of Gs morph adult colonies (Nakaema and Hidaka, 2015). The larvae of *A. millepora* have also been shown to have different coloration from their parents (Kenkel et al., 2011).

When juveniles were inoculated with *Symbiodinium*, the GFP distribution pattern remained unchanged. However, inoculation success was variable depending on the clade type of the *Symbiodinium*. Clade D *Symbiodinium* appeared to proliferate in juveniles, while clade C *Symbiodinium* did not. Thus, it was difficult to analyze the effect of *Symbiodinium* inoculation on GFP expression in juveniles.

This study suggests a possible maternal effect on larval GFP intensity in *G. fascicularis*, as GFP intensity was higher in larvae or juveniles derived from B morph eggs than in those derived from Gs morph eggs. This phenomenon is unexpected, since B morph colonies contain much less GFP than do Gs morph colonies. Future studies are necessary to understand the mechanisms that drive this maternal effect.

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References

Abe, M., T. Watanabe, H. Hayakawa & M. Hidaka. 2008. Breeding experiments of hermatypic coral *Galaxea fascicularis*: partial reproductive isolation between colonies of different nematocyst types and enhancement of fertilization success by presence of parental colonies. Fisheries Science, 74: 1342-1344.

DeSalvo, M.K., C.R. Voolstra, S. Sunagawa, J.A. Schwarz, J.H. Stillman, M.A. Coffroth, A.M. Szmant, & M. Medina. 2008. Differential gene expression during thermal stress and bleaching in the Caribbean coral Montastraea faveolata. Molecular Ecology, 17: 3952–3971.

D'Angelo, C., A. Denzel, A. Vogt, M. Matz, F. Oswald, A. Salih, G. Nienhaus & J. Wiedenmann. 2008. Blue light regulation of host pigment in reef-building corals. Marine Ecology Progress Series, 364: 97–106.

Hidaka, M. & K. Yamazato. 1985. Color morph of *Galaxea fascicularis* found in the reef around the Sesoko Marine Science Center. Galaxea, 4: 33-35.

Kenkel, C.D., M.R. Traylor, J. Wiedenmann, A. Salih & M. V. Matz. 2011 Fluorescence of coral larvae predicts their settlement response to crustose coralline algae and reflects stress. Proceedings of the Royal Society Biological Science, 278: 2691-2697.

Nakaema, S & M. Hidaka. 2015. Fluorescent protein content and stress tolerance of two color morphs of the coral Galaxea fascicularis. Galaxea, Journal of Coral Reef Studies, 17: 1-11

Rodriguez-Lanetty, M., S. Harii & O. Hoegh-Guldberg. 2009. Early molecular responses of coral larvae to hyperthermal stress. Molecular Ecology, 18: 5101–5114.

Roth, M.S., M.I. Latz, R. Goericke & D.D. Deheyn. 2010. Green fluorescent protein regulation in the coral *Acropora yongei* during photoacclimation. Journal of Experimental Biology, 213: 3644–3655.

Roth, M.S., & D.D. Deheyn. 2013. Effects of cold stress and heat stress on coral fluorescence in reef-building corals. Scientific Reports, 3: 1421.

Salih, A., A. Larkum, G. Cox, M. Kühl & O.Hoegh-Guldberg. 2000. Fluorescent pigments in corals are photoprotective. Nature, 408: 850–853.

Shinzato, C., E. Shoguchi, M. Tanaka & N. Satoh. 2012. Fluorescent protein candidate genes in the coral *Acropora digitifera* genome. Zoological Science, 29: 260–264.

Smith-Keune, C. & S. Dove. 2007. Gene expression of a green fluorescent protein homolog as a host-specific biomarker of heat stress within a reef-building coral. Marine Biotechnology, 10: 166–180.

Takabayashi M & O. Hoegh-Guldberg. 1995. Ecological and physiological differences between two colour morphs of the coral *Pocillopora damicornis*. Marine Biology, 123: 705–714.

Yuyama I, S. Harii & M. Hidaka. 2012. Algal symbiont type affects gene expression in juveniles of the coral *Acropora tenuis* exposed to thermal stress. Marine Environment Research, 76: 41-47.