5-2012

Optimization of a Fluorescent In Situ Hybridization Protocol in the Chloroplast Hosting Mollusc Elysia chlorotica

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OPTIMIZATION OF A FLUORESCENT IN SITU HYBRIDIZATION PROTOCOL IN THE CHLOROPLAST HOSTING MOLLUSC ELYSIA CHLOROTICA

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biochemistry)

The Honors College

University of Maine

May 2012

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Abstract

The symbiosis between the mollusc *Elysia chlorotica* and chloroplasts of its algal prey, *Vaucheria litorea*, has challenged the understanding of chloroplast biology. *E. chlorotica* feeds on and retains the algal chloroplasts within its digestive cells. The chloroplasts remain photosynthetically active for months in the animal's cells devoid of the algal nucleus which encodes approximately 90% of the proteins required for the chloroplasts. To help explain the ability of *E. chlorotica* to maintain functionally active chloroplasts, horizontal gene transfer (HGT) of *V. litorea* nuclear genes to the *E. chlorotica* genome has been hypothesized. However, the extent of HGT, as well as the transcriptional viability of putatively transferred genes, is still largely unknown. To better understand the genetic composition of *E. chlorotica*, optimization of a fluorescent *in situ* hybridization (FISH) procedure was performed on both *V. litorea* and *E. chlorotica* to develop a direct molecular tool to understand the genetic components involved in this symbiosis. Successful FISH analysis of the chloroplast Rubisco gene (*rbcL*) was accomplished using whole-mounts of both *E. chlorotica* and *V. litorea*. Whole-mount FISH analysis of the *E. chlorotica* nuclear β-actin gene was accomplished in aposymbiotic *E. chlorotica* larvae. Sectioned, adult *E. chlorotica* tissue was successfully hybridized for both the *rbcL* and *β-actin* genes. The development of the FISH technique for use on whole-mount sea slugs and intact heterokont algal filaments is the first direct application of FISH and can help elucidate the genetic components required to maintain this remarkable symbiosis.

Acknowledgements

I would like to thank Dr. Mary Rumpho for allowing me the opportunity to do research in her lab. Through her mentorship, I have found a burgeoning interest in the dynamics of plant biology as well as a greater understanding of the connectedness of different scientific disciplines.

I would like to thank Dr. Karen Pelletreau for taking a first-year biochemistry student who did not know how to use a micropipette and helping me develop the skills necessary to do quality bench work.

I would like to thank Susan Devine and Vanessa Coats for not only sharing an office with me, but also as two collaborative resources in the obstacles that presented themselves in the completion of my research.

I would like to thank Kathryn Dutil, Kara Sarver, and Susan Devine for their assistance in the maintaining the algal culture in the lab.

I would like to thank Dawna Beane of the University of Maine Animal Health Laboratory for embedding and sectioning *E. chlorotica* samples.

This research was supported in part by a National Science Foundation (grant #IOS-0726178) and Hatch Project no. ME08361-08MRF (NC1168) to M.E.R. and an Honors Thesis Fellowship supported by grants from the National Center for Research Resources (5P20RR016463-12) and the National Institute of General Medical Sciences (8 P20 GM103423-12) from the National Institutes of Health to G.A.D.

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Literature Review

E. chlorotica – V. litorea symbiosis.

The long-term sequestration of functional algal chloroplasts by sacoglossan molluscs or sea slugs (referred to as kleptoplasty) provides a remarkable example of modern endosymbiosis evolution. The green, "solar-powered" sea slug *Elysia chlorotica* Gould, 1870, has been one of the most studied examples of long-term kleptoplasty (reviewed by Rumpho et al. 2011). During feeding on the siphonaceous, coenocytic heterokont alga *Vaucheria litorea* C. Agardh, *E. chlorotica* suctorally ingests the cellular components of the algal filament (Trench 1975). *V. litorea* chloroplasts are not digested upon ingestion, but become incorporated, most likely through phagocytosis, into the cells lining the digestive tract of *E. chlorotica*. The chloroplasts remain within the cytosol of these cells for the duration of the mollusc's lifespan, conferring a new metabolic capability (photosynthesis) to the sea slug (Gibson et al. 1986, Green et al. 2000). *E. chlorotica* can be sustained in laboratory culture under starvation conditions for 9-12 months, living photoautotrophically on the photosynthate of the chloroplasts within its digestive cells (Green et al. 2000). This chloroplast association is not transferred vertically, rather, chloroplast-free eggs are deposited by green, photosynthetically active adult sea slugs and develop through a planktonic veliger stage in which the larvae selectively filter feed on unicellular algae prior to metamorphosis (Harrigan and Alkon 1978; Trowbridge 2000). Upon reaching metamorphic competentency, exposure to *V. litorea* induces metamorphosis, with veligers crawling out of their shells and immediately incorporating the chloroplasts into their digestive cells after feeding on *V. litorea* (West et al. 1984; Pelletreau *unpublished data*).

Horizontal gene transfer between multicellular eukaryotes.

Although the chloroplasts do not divide in the sea slug, *de novo* protein synthesis (of both chloroplast and algal nuclear genes) and carbon dioxide fixation continue in the absence of the algal nucleus, upon which the chloroplasts are dependent for algal nuclear encoded proteins (Mujer et al. 1996; Pierce et al. 1996; Rumpho et al. 2008, 2009). In order to provide key photosynthetic transcripts for the chloroplasts, horizontal gene transfer (HGT) from the algal nucleus to the sea slug nucleus has been indirectly demonstrated using mainly PCR analysis investigating algal nuclear-encoded photosynthesis genes (Hanten and Pierce 2001; Pierce et al. 2003, 2007, 2009; Rumpho et al. 2008, 2009; Schwartz et al. 2010). However, recent transcriptome analysis of kleptoplastic *Elysia* sp. has indicated a varying extent of transcripts produced from putative HGT genes. For example, no algal nuclear related transcripts were found in initial and partial transcriptome studies of adult *E. chlorotica* (Rumpho et al. 2011) or *E. timida* and *Plakobranchus ocellatus* (Wägele et al. 2011). In a second transcriptome study of *E. chlorotica* concurrent with sequencing the *V. litorea* genome, 52 transcripts were identified in the *E. chlorotica* transcriptome matching algal nuclear genes, 27 of which were related to chloroplast metabolism and function (Pierce et al. 2012).

Fluorescent in situ hybridization.

The ambiguity of potential HGT events in *E. chlorotica* necessitates the use of a more direct molecular approach to investigate the genomic content of the sea slug. With the development of non-isotopic *in situ* hybridization techniques, the ability to utilize labeled DNA or RNA probes has been applied to numerous systems. After fixing a

sample in a buffered fixative that ensures proper retention of cellular nucleic acid locations, samples are incubated with a tagged nucleic acid probe under conditions that favor the formation of probe-target sequence duplexes. Commercially available probes have been developed for target sequences routinely examined, such as bacterial 16S probes and probes covering all human and mouse chromosomes. Novel probes are routinely synthesized by investigators for a sequence of interest, through either direct incorporation of a labeled nucleotide triphosphate or by secondary labeling of a modified nucleic acid backbone. Successful hybridization of probe(s) to target sequences can then be detected with fluorescent or colorimetric techniques. The advantages of using sequence specific probes designed to hybridize to a sequence of interest allows direct access of the target sequence.

The application of *in situ* hybridization can be modified to answer a varied range of questions. Bacterial symbionts can be identified at various taxonomic levels depending upon the specificity of the probes used (Kaiwa et al. 2011), as well as algal symbionts in both vertebrate (Kerney et al. 2011) and invertebrate hosts (Loram et al. 2007). FISH has been used to identify HGT events of the intracellular bacterium *Wolbachia* to host arthropods (Aikawa et al. 2009; Nikoh et al. 2008), of a diatom freeze protection gene to the crustacean *Stepho longipes* (Kiko 2010), and of a poly-glutamate synthase gene in cnidarians (Denker et al. 2008). Few studies, however, have usdd FISH to identify targets in molluscs. Cytogenetic FISH studies using isolated metaphase chromosomes of marine molluscs have been used determine chromosome structure (Vitturi et al. 2000a, 2000b), and developmental expression of genes relating to neurogenesis were identified in veliger larvae of *Gibbula varia* (Samadi and Steiner 2010) and *Aplysia californica* (Heyland et

al. 2010), but few studies have used adult mollusc tissues. To localize DNA sequences in *E. chlorotica* and its algal prey *V. litorea*, a protocol for FISH was optimized to identify genes in both the chloroplast genome (Rumpho et al. 2008) and the sea slug nuclear genome. Utilizing DNA probes for potential HGT genes, FISH could help elucidate the extent of any HGT events as well as what genes are present together within the *E. chlorotica* genome.

Materials and Methods

Algal culturing conditions.

Unialgal cultures of *V. litorea* and *Isochrysis galbana* were cultured in artificial sea water (ASW; Instant Ocean©) at 32 PSU at 24ºC supplemented with a modified f/2 medium on a 12:12 L:D cycle. *V. litorea* cultures were subcultured biweekly in progressively lower (16 and 8 PSU) salt concentrations, followed by an increase in salt concentration (16 and 32 PSU).

Animal collection and maintenance.

Wild *E. chlorotica* were collected from populations in Martha's Vinyard, MA, in November, 2011, and maintained at 10ºC in aerated ASW at 32 PSU on a 12:12 L:D cycle. To induce egg laying, 4-6 animals were removed to 24ºC and placed in 1 L of ASW with access to *V. litorea*. Egg ribbons were collected immediately, rinsed thoroughly in 0.2 μm filtered autoclaved ASW (faASW) and transferred to 500 ml of faASW supplemented with 2.5 mg ml^{-1} of chloramphenicol (Capo et al. 2009). After hatching, *E. chlorotica* veliger density was determined using a Nikon SMZ-800 stereomicroscope and maintained in faASW at a density of 10 veliger ml^{-1} and fed

unialgal cultures of *I. galbana* daily at a concentration of 1 x 10^5 cells ml^{-1}. Cultures were washed twice a week by filtering the veligers through a 50 μm Nytex mesh and gently spraying with faASW before being returned to fresh faASW supplemented with chloramphenicol (2.5 mg ml^{-1}). The development of veligers was observed twice weekly until physical markers of metamorphic competency (dark pigment on the caudal tip, and a loss of velum) were obtained. Competent veligers were collected by straining through a 120 μm Nitex mesh, gently rinsed by spraying with faASW, and transferred to culturing dishes containing 500 ml of faASW and *V. litorea* to induce metamorphosis. Successfully metamorphosed F2 *E. chlorotica* were allowed to feed on *V. litorea* for one month at 24ºC and subsequently maintained at 10ºC on a 12:12 L:D cycle in aerated ASW and allowed to feed on *V. litorea ad libitum* (Pelletreau *unpublished data*).

Chloroplast isolation.

Chloroplasts were isolated from *V.* litorea filaments following Rumpho et al. 2009 as follows. Algae (~0.5 g wet weight) was homogenized in 50 ml of ice-cold grinding buffer (GB; 0.1 M 4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid [HEPES]/KOH, 0.9 M sorbitol, 2 mM $MgCl_2$, 4 mM Na_2- ethylenediaminetetraacetic acid [EDTA], 10 mM Na ascorbate, 2% BSA; pH 7.5). The filtered homogenate was centrifuged in a Beckman JA 25.5 rotor at 1200*g* for 5 min (4ºC) and the pellet resuspended in 5 ml of GB and filtered through a 20 μm Nytex mesh. The chloroplast extract was layered on top of a 25%/75% Percoll gradient and centrifuged for 20 min at 8000*g* (4ºC) using a JA 25.5 rotor. Chloroplasts at the interphase were collected by aspiration and washed twice in GB without BSA and centrifuged at 1500*g* for 5 min (4ºC) in a JA 25.5 rotor. The final

chloroplast pellet was dissolved in resuspension buffer (0.1 M HEPES, 0.45 M sorbitol, 10 mM $MgCl_2$) and stored protected from light at 4ºC. Chloroplasts were examined by phase contrast microscopy prior to hybridization to determine the relative percentage of chloroplasts with intact membranes.

DNA isolation.

Genomic DNA was extracted from *V. litorea* and *E. chlorotica* using DNAzol (Molecular Research Center, Inc., Cincinnati, OH, USA) isolation protocol. *V. litorea* and *E. chlorotica* samples (~0.2 g fresh weight each) were ground in liquid nitrogen and homogenized in DNAzol isolation reagent (3:1 volume:weight). Organic solutes were removed with the addition of 1% mercaptoethanol and chloroform (1:1 chloroform:DNAzol) and rocked gently for 15 min at 24ºC. Samples were centrifuged at 12,000*g* for 10 min. The DNA was precipitated with 100% ethanol at -20ºC, followed by a second chloroform extraction. DNA was precipitated with the addition of 3 M sodium acetate and cold 100% ethanol. The precipitated DNA was collected by centrifugation at 12,000*g* for 10 min. The pellet was washed with 75% ethanol and suspended in LoTE buffer (3 mM Tris-HCl, 0.2 mM EDTA; pH 8.0). DNA concentration was determined using a NanoDrop 100 spectrophotometer (Thermo Scientific).

DNA cloning and sequencing.

The chloroplast control probe for the *V. litorea* Rubisco large subunit (*rbcL*) was amplified from *V. litorea* DNA by PCR to produce an 892 base pair (bp) probe with primers 5’CCTTAATACAACTGCAG and 5’CCTTTATTTACAGCATAC (Green et al.

2000). A 160 bp probe for *E. chlorotica* β-actin was generated using the primers 5'ACGAGGCTCAGTCCAAGAGA and 5'GGAGCCTCTGTGAGCAAGAC. PCR conditions used to amplify each gene included 1X enzyme reaction buffer (Promega, Madison, WI, USA), 25 mM $MgCl_2$, 10 mM deoxynucleotide triphosphate (dNTP) mix, forward and reverse primers (10 μM each), and 1.25 U GoTaq® DNA Polymerase (Promega). Cycle conditions were: 94ºC for 2 min, 40 cycles of 94 ºC for 1 min, 49 ºC for 1 min, and 72 ºC for 1 min followed by a final elongation at 72 ºC for 10 min. PCR products were separated using a 1% agarose gel in TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA) and visualized by ethidium bromide staining and UV transillumination. PCR products were stored at -20 ºC for no more than 24 hr before cloning into the pGEM-T Easy Vector System (Promega). Plasmids containing *rbcL* and *β-actin* were sequenced at the University of Maine DNA Sequencing Facility with SP6 primers to ensure sequence identity.

Fluorescent probe synthesis.

DNA for generating fluorescent probes was generated by PCR amplification of transformed *rbcL* plasmid with *rbcL* forward and reverse primers and the *β-actin* plasmid with *β-actin* forward and reverse primers. FISH probes were synthesized from 1 μg of amplified DNA using the FISH Tag™ DNA Multicolor Kit (Invitrogen) according to the manufacturer's specifications. *RbcL* probes were labeled with AlexaFluor® 488 (excitation/emission 488/519 nm) and *E. chlorotica* β-actin probes were labeled with either AlexaFluor® 555 or AlexaFluor® 594 (excitation/emission 555/565 nm and

590/615 nm, respectively). Probes were stored protected from light at -20ºC and used within 2 weeks of synthesis.

Sample preparation for in situ hybridization.

All sea slug and algal samples for FISH analysis were fixed in 4% paraformaldehyde in PIPES buffer (50 mM piperazine-N,N′-bis(2-ethanesulfonic acid) [PIPES], 100 mM KCl, 2 mM EDTA, 10 mM ethylene glycol tetraacetic acid [EGTA], 5 mM $MgCl_2$, 200 mM NaCl, 5% dimethyl sulfoxide) at 4ºC overnight (Mine et al. 2001). Isolated chloroplasts were fixed the same day that they were isolated to obtain intact chloroplasts for hybridization. *E. chlorotica* F2 generation animals were anesthetized in ASW containing 7% (w/v) $MgCl_2$ to allow the parapodia to be fixed in an open body position. *E. chlorotica* veligers hatched from chloroplast-free eggs were collected within 24 hrs after release from the egg ribbon and fixed prior to any feeding. Chloroplasts and veligers were collected by centrifugation at 5000*g* following fixation and each buffer treatment. Veligers were processed post-fixation to remove the shells by washing 3 times for 10 min in PIPES buffer followed by digestion with 0.5% trypsin for 15 min. Actively growing *V. litorea* filaments were rinsed briefly in deionized water and cut into ~2 cm long segments before fixation. All samples were washed 3 times for 10 min each in PIPES buffer after fixation and dehydrated in a graded ethanol series from 70% to 100% and stored at -20ºC until hybridization.

RbcL in situ hybridization.

Fixed *V. litorea* filaments, isolated chloroplasts, and *E. chlorotica* F2 generation samples were removed from -20ºC and rehydrated in 3 washes with PIPES buffer at 24ºC and equilibrated in prehybridization buffer (25% formamide in PIPES buffer) for 10 min at 24ºC. Hybridization solution (50% formamide in PIPES buffer, 1 μg ml^{-1} salmon sperm DNA) containing either 100 or 200 ng of labeled *rbcL* probe DNA was added and samples heated in a 95ºC water bath for 5 min to denature probe and target DNA. Hybridization was carried out overnight at 40ºC followed by a high stringency wash in post-hybridization buffer (50% formamide in PIPES buffer) at 45ºC for 10 min, and 3 washes for 10 min each in PIPES buffer. Samples were viewed using an Olympus Fluoview® Confocal Microscope equipped with AlexaFluor® 488 filters (excitation/emission 488/519 nm) to visualize bound *rbcL* probe-gene duplexes and Cy5 filters (excitation/emission 635/664 nm) to visualize chlorophyll autofluorescence. The depth (z-plane) of the microscope was adjusted to include the entire algal filament diameter, and adjusted to include the entire height of digestive diverticula in imaged *E. chlorotica*. Serial 1 μm z-plane sections were compiled into stacked images.

Rehybridization of E. chlorotica and V. litorea filaments.

Samples hybridized with 100 ng of *rbcL* probe were rehybridized immediately after initial viewing with the confocal microscope. Samples were washed 3 times in PIPES buffer for 10 min each and then equilibrated in prehybridization buffer for 10 min. Hybridization solution containing 100 ng of the labeled *rbcL* probe was added to each sample to increase the effective probe concentration, and the samples were heated in a

95ºC water bath for 5 min to denature probe DNA, target DNA, and previously established probe-target duplexes. Samples were hybridized overnight at 40ºC followed by a high stringency wash in post-hybridization buffer at 45ºC for 10 min, and 3 washes for 10 min each in PIPES buffer. Samples were viewed using an Olympus Fluoview® Confocal Microscope equipped with AlexaFluor® 488 filters to visualize labeled *rbcL* probe-gene duplexes and Cy5 filters to visualize chlorophyll fluorescence. Combined z-stacked images were obtained as above.

β-actin in situ hybridization.

E. chlorotica F2 animals and veligers were removed from -20ºC and rehydrated in 3 washes with PIPES buffer at 24ºC and equilibrated in prehybridization buffer for 10 min at 24ºC. Hybridization solution containing either 200 or 400 ng of labeled *β-actin* probe DNA was added to the samples and heated in a 95ºC water bath for 5 min. Hybridization was carried out overnight at 40ºC followed by a high stringency wash in post-hybridization buffer (50% formamide in PIPES buffer) at 45ºC for 10 min, and 3 washes for 10 min each in PIPES buffer. Samples were incubated with DAPI (5 μg ml^{-1}) for 10 min to label DNA followed by 3 washes with PIPES buffer. Samples were viewed using an Olympus Fluoview® Confocal Microscope equipped with DAPI filters to visualize labeled total nuclear DNA regions, AlexaFluor® 594 filters (excitation/emission 543/618 nm) to visualize labled *β-actin* -gene duplexes, and Cy5 filters to visualize chlorophyll autofluorescence. The focal plane of the microscope was adjusted to include the entire veliger body. Compiled z-stacked images were obtained as above.

Sectioned E. chlorotica in situ hybridization

E. chlorotica F2 generation animals were fixed and dehydrated as described above. Sea slugs were embedded in paraffin wax and 10 μm longitudinal sections were prepared by the University of Maine Animal Health Lab. Slides containing *E. chlorotica* sections were permeabilized with proteinase K (5 μg ml^{-1}) in reaction buffer (2 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$) at 37ºC for 10 min. Proteinase digestion was stopped by rinsing slides with reaction buffer containing 50 mM $MgCl_2$ followed by two washes in PIPES buffer for 5 min. Slides were post-fixed in PIPES buffered 4% paraformaldehyde for 10 min at room temperature and washed 3 times with PIPES buffer. AlexaFluor® 488 tagged *rbcL* and AlexaFluor® 555 *β-actin* probes were denatured for 5 min in a 95ºC water bath and added to hybridization buffer. Hybridization mixture containing denatured probes was added to slides at a final concentration of 200 ng of each labeled probe per slide. Slides were heated on a heatblock at 75ºC for 10 min followed by slow cooling to 40ºC and overnight hybridization. Slides were washed at 45ºC in post-hybridization buffer for 10 min, and 3 washes for 10 min each in PIPES buffer at room temperature. Slides were incubated with labeled probes and visualized as described above, with AlexaFluor® 568 filters (excitation/emission 543/603 nm) to visualize *β-actin* hybridization. The focal plane of the microscope was adjusted to include the entire depth of each section imaged. Sections were scanned with a single excitation wavelength at a time and the images from each filter set compiled using NIH Image J (Rasband 1997).

Results

In situ hybridization to the control chloroplast gene rbcL.

Chloroplasts isolated from *V. litorea* were hybridized with the 892 bp *rbcL* AlexaFluor® 488 probe. *In situ* hybridization was limited to areas within isolated chloroplasts (Fig. 1A, green). Analysis of compiled depth field images (z-stacks) of fluorescence from isolated chloroplasts revealed *rbcL* hybridization at multiple levels of depth within the chloroplasts and not a single location. When *rbcL* was hybridized to whole-mount *E. chlorotica* and *V. litorea* samples (at a probe concentration of 100 ng) minimal probe fluorescence was detected (Figs. 2 and 3, respectively). *RbcL* fluorescence was minimal, with the majority of chloroplasts did not contain any *in situ rbcL* signal. Samples of both *E. chlorotica* and *V. litorea* initially hybridized with 100 ng of *rbcL* probe were serially hybridized immediately after examining for *in situ* signals with a second treatment with 100 ng of *rbcL* probe. The increased effective probe concentration produced a greater *in situ rbcL* fluorescent signal. The chloroplast morphology of the algal filaments as well as nearly ubiquitous chlorophyll fluorescence indicated lysis of chloroplasts during the *in situ* protocol. Serially hybridized *E. chlorotica* samples exhibited foci of FISH signal colocalization with chlorophyll fluorescence (Figs. 2F, yellow). Serially hybridized *V. litorea* filaments also had a stronger *in situ rbcL* signal following the second hybridization, but displayed a greater loss of tissue integrity following multiple denaturation steps (Figs. 3F, yellow).

Whole-mount in situ *hybridization of* β-actin.

Whole *E. chlorotica* veligers were hybridized with the 160 bp *β-actin* probe (Fig. 4). Post-hybridization of the veliger nuclei with the DNA binding fluorophore DAPI (Fig. 4A) allowed the identification of nuclear regions within the newly hatched veliger's cells. The *β-actin* FISH fluorescent signal (Fig. 4B) was detected in regions which also stained with DAPI (Fig. 4C, white), indicating successful hybridization of the *β-actin* probe to the *E. chlorotica* nuclear gene. Little background fluorescent signal was detected in any non-nuclear regions of the veligers. Adult *E. chlorotica*, however, had no specific FISH signal from the labeled *β-actin* probe (Fig. 5). Fluorescence from the labeled *β-actin* probe (Fig. 5B) had no specific localization in *E. chlorotica* samples and minimal colocalization with DAPI labeled *E. chlorotica* DNA (Fig. 5C).

FISH to sectioned E. chlorotica samples.

Sectioned *E. chlorotica* samples were hybridized with labeled *rbcL* and *β-actin* probes (Fig. 6). FISH signal could be detected for both *β-actin* (Fig. 6B) and *rbcL* probes (Fig. 6C). The FISH signal for *β-actin* was isolated to DAPI labeled *E. chlorotica* nuclear DNA (Fig. 6D, white). Chlorophyll autofluorescence was not observed in any of the chloroplasts in the fixed and sectioned tissues, however alignment of the *rbcL* FISH signal with the DIC overlay showed successful hybridization to chloroplasts of the digestive diverticula with the *rbcL* probe (Fig. 6E).

Discussion

The development of a FISH protocol to further characterize HGT in the kleptoplastic sea slug *E. chlorotica* could allow greater insight into the mechanisms of

chloroplast maintenance and long-term photosynthetic activity in the absence of the host algal nucleus. While studies using PCR to identify photosynthetic genes of interest have identified putative HGT events, the discrepancy between PCR studies and large-scale transcriptome analysis highlights the importance of developing a wider toolkit for the understanding of this remarkable symbiosis.

FISH analysis of the Rubisco large subunit gene in *V. litorea* chloroplasts within both *E. chlorotica* and the alga are the first instances of successful applications of FISH to either organism. Localization of *in situ* signal to multiple depths of the spherical, isolated *V. litorea* chloroplasts is consistent with the known presence of multiple copies of the chloroplast genome in chloroplasts (Choquet and Wollman 2002). While FISH analysis of plant samples is often used with non-chloroplast tissue due to interfering chlorophyll fluorescence, the chloroplasts in *E. chlorotica* can be used as qualitative markers of tissue integrity when optimizing the hybridization steps during FISH. The ubiquitous distribution of chlorophyll autofluorescence in *V. litorea* filaments suggests tissue degradation during the *in situ* protocol, necessitating more work in the necessary fixation procedure needed for stable *V. litorea* filaments.

The ability to access the nuclear genome of *E. chlorotica* using FISH is of particular interest to better understand the role of HGT in the maintenance of captured chloroplasts. FISH probing of *E. chlorotica* veligers with fluorescent probes specific for *E. chlorotica* β-actin allowed the FISH signal to be observed in the nuclei of newly hatched veligers. While the veliger stage of development is aposymbiotic, genes that have been horizontally transferred to *E. chlorotica* and stably integrated into the nuclear genome should be accessible targets for FISH probing in veligers. The small size of the

newly hatched veligers allows visualization of most of the animal nuclei in one viewing field, making FISH a powerful tool to visualize HGT related genes to determine if they have been successfully integrated into the *E. chlorotica* germline. While the veliger stage is aposymbiotic, transcriptional profiling of *E. chlorotica* development using FISH with RNA probes can help elucidate the roles of potential transcripts required in the establishment of chloroplast symbiosis. In the neurological model organism *Aplysia californica*, mRNA-FISH was combined with microarray profiling to elucidate developmental transcription and localization of transcripts related to neurological development as well as metamorphic competence (Heyland et al. 2010). The utilization of FISH as a direct molecular approach to target genes of interest in *E. chlorotica* could allow the building of a better understanding of the genomic stability as well as the transcriptional profile in a non-model organism without a currently sequenced genome.

FISH analysis of adult *E. chlorotica* using the fluorescent β-actin DNA probe did not produce a successful FISH signal in the adult whole-mount sea slugs. While the *rbcL* probe was successful in penetrating the sea slug tissue and FISH signal was detected in chloroplasts, the *β-actin* fluorescence was diffuse throughout the cells. Potentially, copies of the β-actin transcript could have been left undegraded in the cytoplasm of *E. chlorotica* cells, and binding of similar sequences with the *β-actin* probe to cytoplasmic transcripts could have limited the ability of the probe to produce specific a FISH signal.

In sectioned, adult *E. chlorotica* tissue, both the Rubisco and β-actin genes were able to be successfully hybridized to the chloroplast and sea slug nuclear genome, respectively. FISH detection of *rbcL* in sectioned material produced a more uniform labeling of chloroplasts compared to whole-mount material, with FISH signal similar to

that seen in isolated chloroplasts. While chlorophyll autofluorescence could not be detected in sectioned samples, chloroplasts could be visualized in the DIC image of the sectioned material, which also matched the location of the FISH *rbcL* signal. FISH detection of *β-actin* was accomplished in the sectioned *E. chlorotica* adult, however multiple foci appeared within the DAPI labeled *E. chlorotica* nuclear DNA. These multiple FISH *β-actin* signals could be due to multiple copies of the β-actin gene in the *E. chlorotica* genome, as 12 actin isoforms are present in the gastropod *Placopecten magellanicus* (Patwary et al. 1996) and 4 isoforms in the gastropod *Patella vulgata* (Van Loon et al. 1993).

Development of a fluorescent *in situ* hybridization technique to use with *E. chlorotica* and *V. litorea* would provide a powerful cytological tool to better understand the genetic mechanisms supporting this long-term, functional chloroplast symbiosis. The inconsistencies between HGT studies and transcriptome sequencing experiments necessitate the use of more techniques in order to draw conclusions on the genetic basis of plastid maintenance in an animal. The ability to successfully identify the *E. chlorotica* nuclear β-actin gene in both veliger and sectioned *E. chlorotica* nuclear DNA using FISH provides an opportunity for the technique to be employed on other nuclear genes of interest. Application of FISH to HGT studies using both *V. litorea* and *E. chlorotica* can help determine the extent of HGT in this symbiosis. While DNA probes were used in this study, the protocol for FISH in *E. chlorotica* and *V. litorea* could be applied to RNA probes to identify the transcripts of HGT genes. The combined application of using DNA-FISH to identify HGT genes as well as RNA-FISH to identify HGT transcripts could provide the foundation for how to employ larger sequencing studies.

Figures

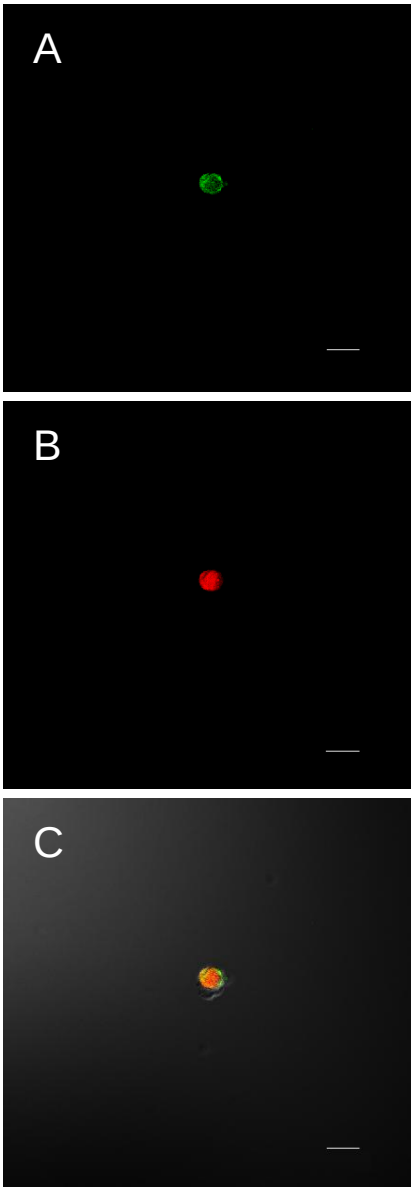

Figure 1: The Rubisco large subunit (*rbcL*) gene was successfully hybridized in *V. litorea* isolated chloroplasts. (A) Intact, isolated chloroplasts were successfully hybridized with AlexaFluor® 488 (green) tagged *rbcL* probe. (B) Chlorophyll autofluorescence (red) was visualized with a Cy5 filter set. (C) Merged *rbcL in situ* signal and chlorophyll fluorescence channels with DIC overlay show successful probe penetration of isolated chloroplasts by the *rbcL* probe and hybridization to the *V. litorea* *rbcL* gene. (Scale bar = 10 μm)

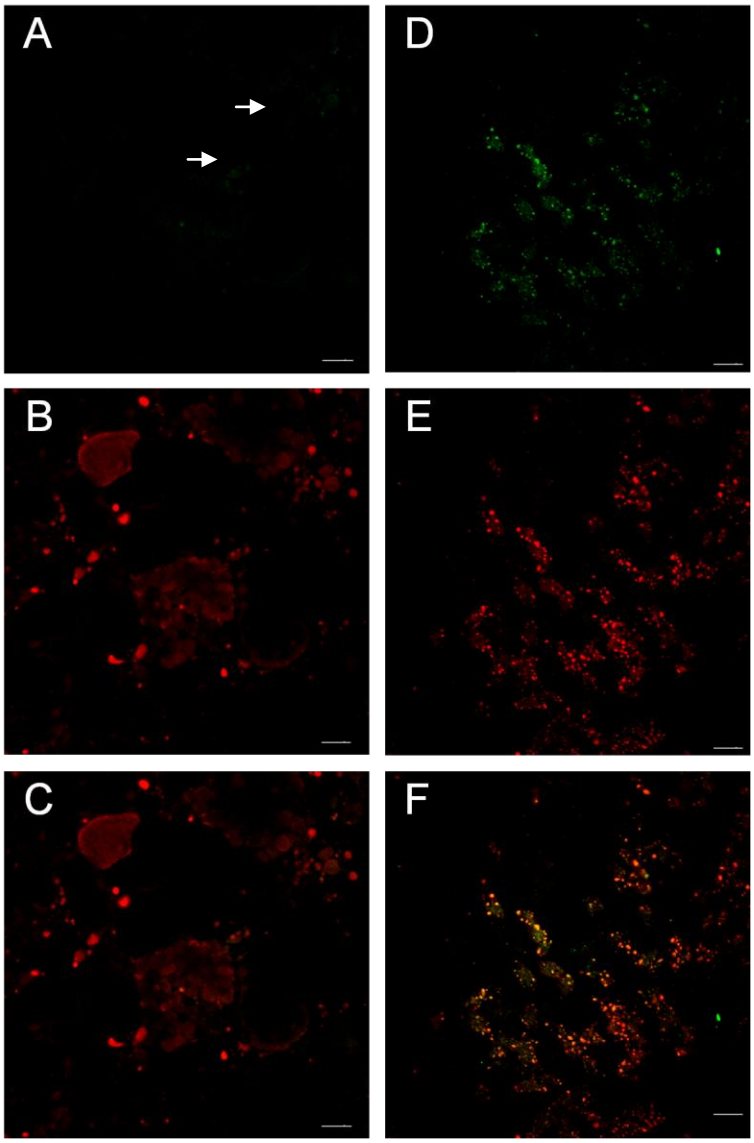

Figure 2: Whole-mount *E. chlorotica* FISH targeting of the chloroplast Rubisco (*rbcL*) gene. (A-C) *E. chlorotica* hybridized with 100 ng of AlexaFluor® 488 tagged *rbcL* probe had little FISH signal. (A) Minimal *rbcL* signal was seen in sea slugs hybridized with 100 ng of probe (green, arrows). (B) Chlorophyll fluorescence (red) could be visualized in *E. chlorotica* diverticula using a Cy5 filter set. (C) Merged *rbcL* hybridization and chlorophyll channels. Few chloroplasts show successful *in situ* labeling of the *rbcL* gene. (D-F) *E. chlorotica* serially hybridized with AlexaFluor® 488 tagged *rbcL* probes had a greater degree of *in situ* hybridization. (D) *RbcL in situ* hybridization had a high degree of *in situ* signal (green) with minimal background labeling following the second

hybridization. (E) Intact chloroplasts (red) within the diverticula were still present after a second hybridization. (F) Merged *rbcL in situ* signal and chlorophyll channels show chloroplasts containing successfully hybridized *rbcL* genes (yellow). (Scale bar = 10 µm)

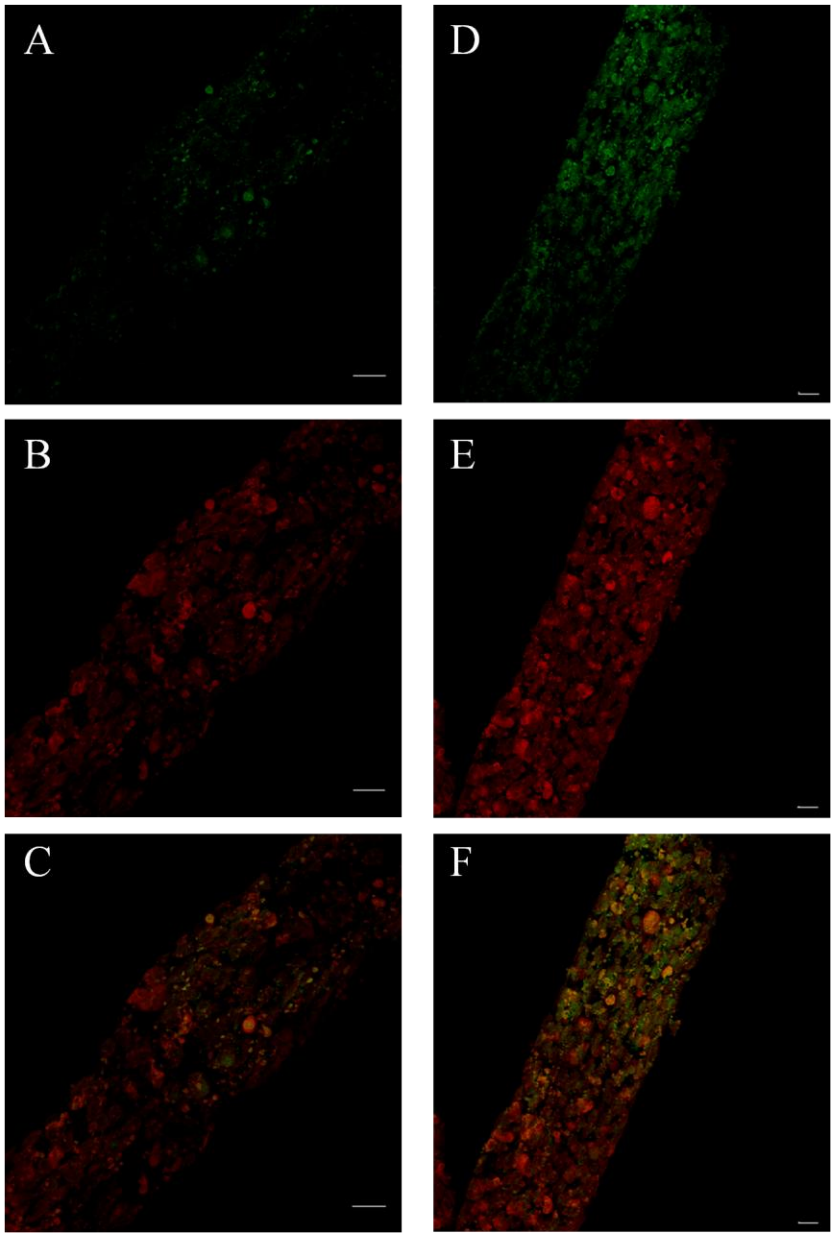

Figure 3: Whole-mount FISH analysis of *V. litorea* filaments targeting the chloroplast Rubisco (*rbcL*) gene. (A-C) *V. litorea* filaments hybridized with 100 ng of AlexaFluor® 488 (green) tagged *rbcL* probe. (*A*) *RbcL* FISH signal was seen in filaments hybridized with 100 ng of probe. (B) Intact chloroplasts can be visualized in *V. litorea* filaments

using a Cy5 filter set to view chlorophyll autofluorescence (red). (C) Merged *rbcL* hybridization and chlorophyll channels show few chloroplasts successfully hybridized with the *rbcL* gene (yellow). (D-F) *V. litorea* serially hybridized with AlexaFluor® 488 tagged *rbcL* probe had a greater degree of *in situ* hybridization to the chloroplast gene. (D) *RbcL in situ* hybridization detected with a set had a high degree of *in situ* signal (green) than the first hybridization. (E) Chloroplasts (red) are still present after the second hybridization of the filament, however the stability of the algal tissue appears to have been damaged during the hybridization process due to the diffuse chlorophyll fluorescence. (F) Merged *rbcL in situ* signal and chlorophyll channels show chloroplasts containing successfully hybridized *rbcL* genes (yellow). A greater degree of successful FISH of *rbcL* was accomplished in the second hybridization. (Scale bar = 10 μm)

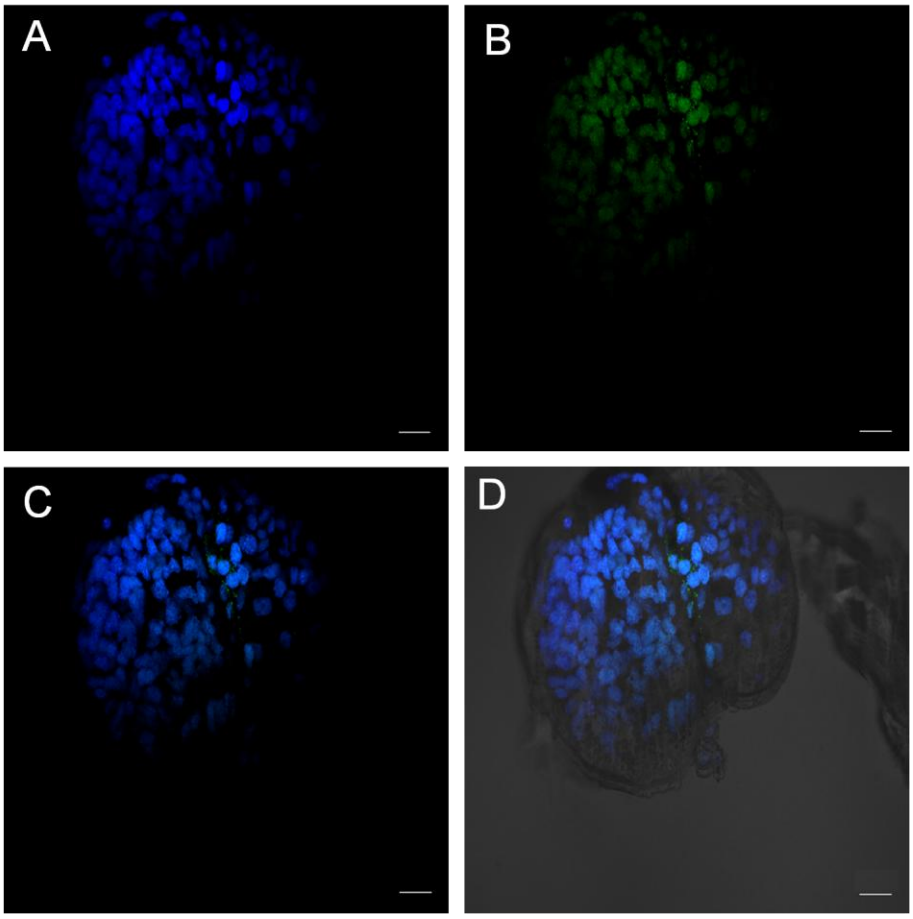

Figure 4: A newly hatched, unfed *E. chlorotica* veliger used for *in situ* hybridization to detect the nuclear β-actin gene. (A) Veliger nuclei were visualized by post-hybridization labeling with DAPI (blue). (B) Veliger probed with AlexaFluor® 594 (green) tagged β-actin had distinct regions of *in situ* signal. (C) Merged DAPI and β-actin channels indicate *in situ* hybridization of the nuclear β-actin gene to *E. chlorotica* nuclei (white) with minimal non-nuclear labeling. (D) DIC overlay of the merged DAPI and β-actin channels allow all nuclei of the veliger to be visualized in a single viewing field. (Scale bar = 10 μm)

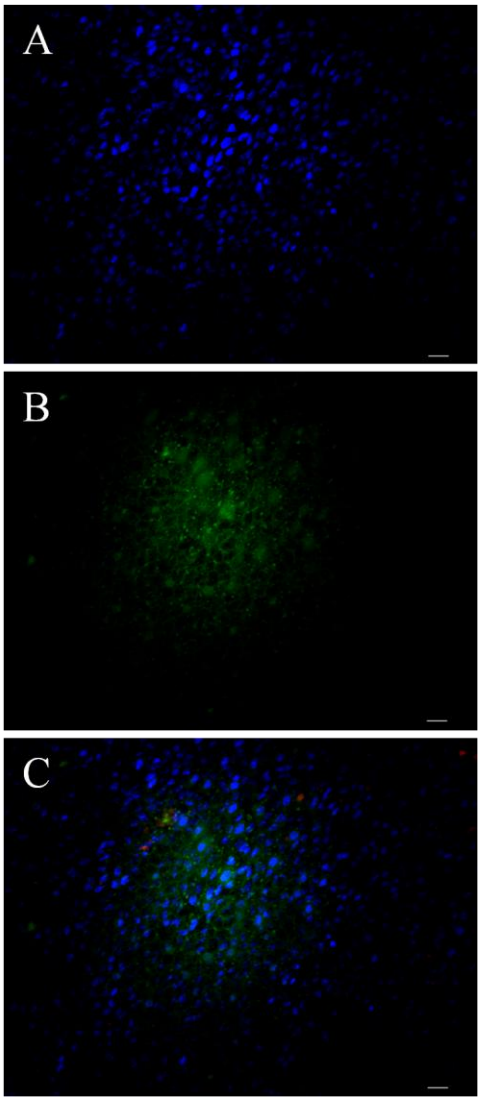

Figure 5: Whole-mount FISH analysis of adult *E. chlorotica* targeting the nuclear β-actin gene did not localize to nuclear regions. (A) Sea slug nuclei were visualized by post-hybridization labeling with DAPI (blue) (B) AlexaFluor® 594 (green) tagged β-actin probes aggregated non-specifically in *E. chlorotica* tissue. (C) Merged DAPI, AlexaFluor® 594, and chlorophyll Cy5 (red) channels show no specific nuclear labeling of sea slug β-actin genes. (Scale bar = 10 μm)

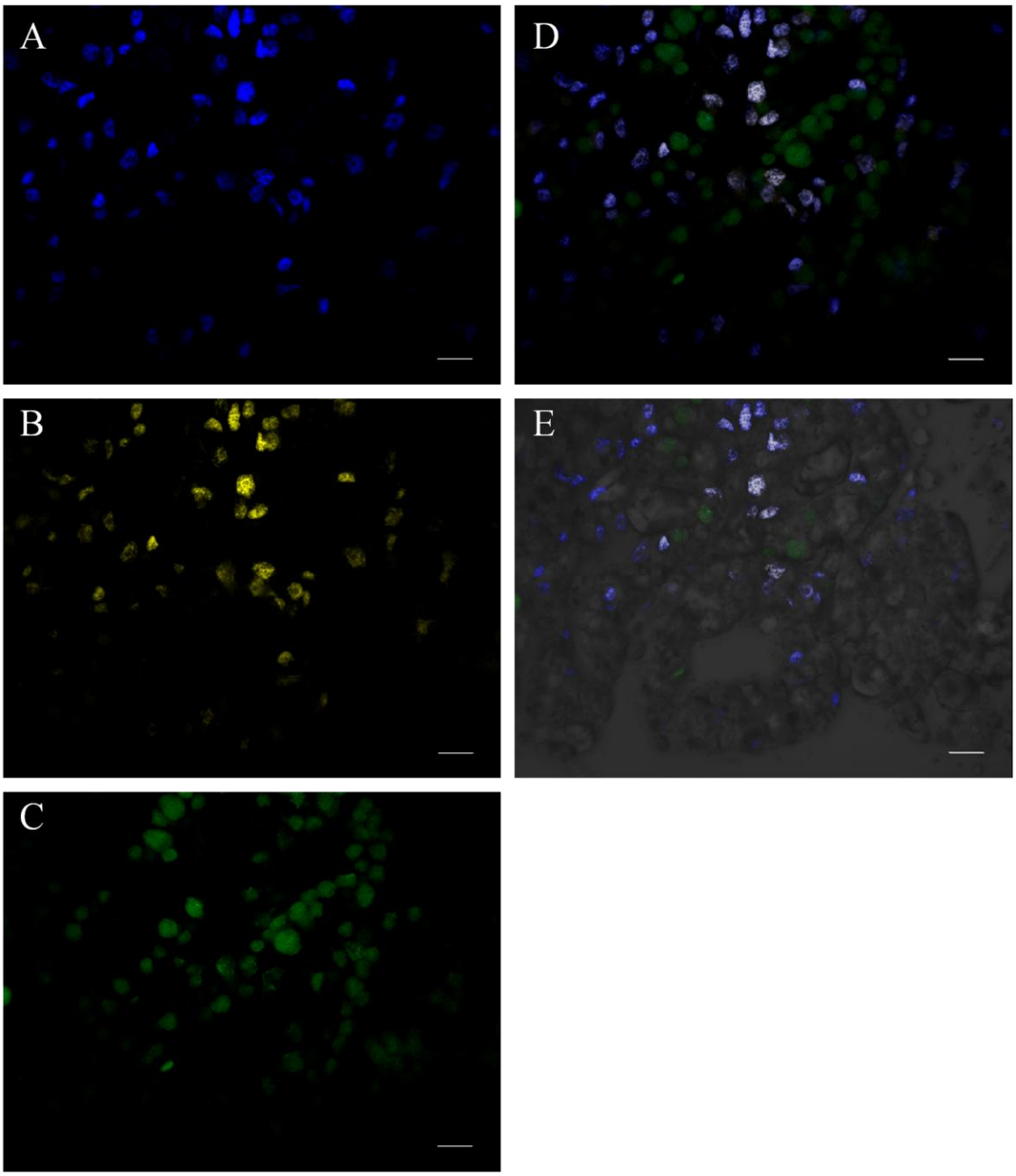

Figure 6: *E. chlorotica* FISH analysis of sectioned adult tissue successfully targeting the nuclear β-actin gene and the chloroplast Rubisco gene. (A) Sea slug nuclei were visualized by post-hybridization labeling with DAPI (blue). (B) AlexaFluor® 568 (yellow) tagged β-actin probe was able to be successfully hybridized to sectioned *E. chlorotica* tissue. (C) AlexaFluor® 488 (green) tagged *rbcL* probe localized exclusively to chloroplast DNA. (D) Merged DAPI, AlexaFluor® 488, and AlexaFluor® 568 images

show the selectivity of each probe for the *rbcL* and *β-actin* genes, respectively. The nuclear *β-actin* gene was successfully hybridized with the *β-actin* probe (white). (E) Intact chloroplasts can be seen in the DIC overlay of merged fluorescent images. No chlorophyll autofluorescence was detectable in the sectioned tissues. (Scale bar = 10 μm)

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