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A Functional Chlorophyll Biosynthesis Pathway Identified in the
Kleptoplastic Sea Slug, *Elysia chlorotica*

by

Julie A. Schwartz

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology
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Dedication

I am dedicating this thesis to my husband, Fran, and my sons, Joel and Matthew.

Without their endless love, support and encouragement I would never have continued this life-changing endeavor to the finish. When I decided to pursue my graduate degree, little did I realize that my entire family would have to experience the rollercoaster ride of ups and downs as well as successes and defeats and I am eternally grateful that they always stayed by my side to help me attain my goal. Our many trips to collect sea slugs and algae and to present research at National and International conferences have been an enjoyable and educational experience for all of us. In fact, I believe that my children are the only ones at their school who could have an intelligent conversation about photosynthetic sea slugs and horizontal gene transfer and could teach others how to use the tblastx logarithm to search databases in NCBI. My words of wisdom to them are that this knowledge may serve them well someday if they ever decide to become a contestant on Jeopardy. Nonetheless, we persevered through all circumstances with love and laughter and have finally made it to the finish line as a team.

Acknowledgments

I would like to express my deepest gratitude to an anonymous donor who believed in the importance of our project and gave continued financial support to our laboratory, so that the research presented in this thesis and other significant discoveries could be made. Without this unwavering support, I would not have been able to complete my research and I will be forever grateful.

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Abstract

The sacoglossan sea slug, *Elysia chlorotica*, feeds upon and sequesters plastids from the heterokont alga, *Vaucheria litorea*, and maintains the metabolically active organelles for up to nine months under starvation conditions while utilizing the photosynthate to survive and reproduce. The photosynthetic pigment, chlorophyll a (Chla), is found in all oxygenic photosynthetic organisms and is responsible for capturing photons of light and converting them into chemical energy. Chlorophyll and its associated proteins involved in the light capturing process are subject to photo oxidative damage and must be continually replaced for ongoing photosynthesis to continue; however, genes encoding these proteins are present in the algal nucleus, presenting a conundrum for sustained plastid photosynthetic activity outside the algal cell. One possibility is that Chla is synthesized by the *E. chlorotica*-kleptoplast association, due to transfer of algal nuclear genes to the sea slug genome. For this study, molecular and biochemical techniques were employed to determine if Chla is synthesized by the animal. Using algal transcriptome sequences for primer design and amplification of target DNA using polymerase chain reaction (PCR), we have identified and sequenced three algal nuclear-encoded gene fragments that correspond to enzymes in the chlorophyll synthesis pathway and one enzyme in the porphyrin synthesis pathway in adult slug and veliger larvae. Sequences from these genes were nearly identical to those present in the alga. Furthermore, these genes are functional; incubation of slugs with radiolabeled 5-aminolevulinic acid (^{14}C -5-ALA), a precursor of chlorophyll biosynthesis, resulted in production of ^{14}C -labeled chlorophyll, as assayed and

identified via HPLC resolution of extracts from slugs. In addition, Chla synthesis in the animal occurs for at least six months under starvation conditions. The discovery of chlorophyll synthesis in *E. chlorotica* is the first animal known to synthesize Chla; moreover, this finding helps elucidate how ongoing photosynthesis can occur in the sea slug after many months in the absence of its algal food.

Introduction

Horizontal gene transfer (HGT), the asexual transfer of genetic material between genetically distinct species, can drive evolution when an organism increases fitness within its environment or new niche by the acquisition of novel genes. HGT mainly occurs amongst prokaryotes (Takeuchi *et al.*, 2014) and prokaryotes and eukaryotes (Keeling and Palmer, 2008; Schönknecht *et al.*, 2013), but is much less prevalent between eukaryotic species due to physical barriers hindering the direct contact of their DNA (Boto, 2014). Although the process is problematic, there is increasing evidence suggesting that lateral transfer may occur between multicellular organisms such as cold water fish (Graham *et al.*, 2008) and certain opisthobranch sea slugs and nuclei from their alga food source to support an endosymbiotic relationship between slug digestive cells and captured algal plastids (Pierce *et al.*, 2007, 2009; Rumpho *et al.* 2008, 2009; Schwartz *et al.*, 2010, 2014).

Sacoglossan sea slugs feed upon the contents of siphonaceous algae and either directly digest the algal matter, or sequester functional chloroplasts into their digestive cells in a process known as “kleptoplasty” (Waugh and Clarke, 1986). The slug’s mechanism for recognition and sequestration of chloroplasts with subsequent digestion of the remaining algal contents is currently not understood. Captured chloroplasts can persist for as little as a few hours in certain species while in others can function for many months under starvation conditions (Pierce and Curtis, 2012). During times of food scarcity, the slugs utilize photosynthate derived from the kleptoplasts to sustain their metabolic processes until food becomes available (Cruz *et al.*, 2013).

Many proteins required to sustain chloroplast metabolism are encoded by algal nuclear DNA, so it is unclear how they remain metabolically active when algal nuclei are not present to supply the necessary proteins (Cruz *et al.*, 2013).

One of the longest persisting endosymbiotic associations occurs between plastids derived from the heterokont alga, *Vaucheria litorea* (C. Agardh, 1823), and the digestive cells of the sacoglossan sea slug, *Elysia chlorotica* (Gould, 1870). The slug slits open the algal filament with its radular tooth, suctorially feeds upon algal cytoplasm and digests all organelles except the plastids. The plastids are sequestered into specialized epithelial cells lining its digestive diverticula (Graves *et al.*, 1979) and remain metabolically active in this foreign environment for remainder of the slug's life [nine months or more] (West, 1979; Pierce *et al.*, 1996).

Furthermore, division of kleptoplasts has never been observed, so captured plastids are not renewed or replaced in the association under starvation conditions (Rumpho *et al.*, 2000).

Although the endosymbiosis in the adult slug persists until the end of its annual life cycle, they do not pass on plastids to their progeny, so the sequestration must occur with each new generation (West, 1979).

Photosynthesis and plastid metabolism requires the complex interaction of many proteins, most of which are encoded by the algal nucleus (Sun, 2009). Plastid photosystem components are in need of continuous replacement due to oxidative damage; Chlorophyll a (Chla) is one such labile component. Chla is found in all photosynthetic organisms and is responsible for the direct transfer of electrons through photosystems II and I, respectively (Masuda and Fujita, 2008).

Chla consists of a magnesium ion containing tetrapyrrole ring and a phytol tail. Tetrapyrrole and chlorophyll synthesis occurs in the plastid and requires enzymes derived from both algal nuclear and plastid encoded genes (von Wettstein *et al.*, 1995). Since Chla molecules are photo damaged

by reactive oxygen species (ROS) produced during the light reactions of photosynthesis, they must be continually replaced (Lyksa *et al.*, 2013; Tyystjarvi, 2013; Serodio *et al.*, 2014); therefore, replacement of damaged Chla is essential for sustained photosynthetic activity in plants and algae.

Since algal nuclei are not captured with the plastids (Pierce *et al.*, 1996), the slug must be utilizing a different source to obtain the necessary proteins for the maintenance of this long-term association. Despite the absence of algal nuclei, transcription of algal nuclear genes in *E. chlorotica* has been detected via amplification of chloroplast-targeted gene fragments from slug cDNA (Pierce *et al.*, 2007, 2009; Rumpho *et al.*, 2008, 2009; Schwartz *et al.*, 2010) and more than 50 algal-nuclear transcripts for plastid metabolism proteins have been identified in the slug transcriptome (Pierce *et al.*, 2012). Chloroplast- and nuclear-encoded protein translation has been detected in *E. chlorotica*. Isolated kleptoplasts from slugs incubated with ³⁵S-methionine exhibited radiolabel incorporation into the chloroplast-encoded proteins, RuBisCO and photosystem II (PSII) protein D1, demonstrating *de novo* synthesis of these crucial proteins (Pierce *et al.*, 1996). In addition, translated slug proteins were radiolabeled with ³⁵S-methionine and several algal nuclear-encoded light-harvesting complex I (LHCI) proteins were identified in *E. chlorotica* as well (Hanten and Pierce, 2001). Lastly, the amplification of algal nuclear-encoded genes from kleptoplast-free veliger DNA indicates that the genes are localized in their genome (Pierce *et al.*, 2007, 2009; Rumpho *et al.*, 2008, 2009; Schwartz *et al.*, 2010).

Many hypotheses have been proposed which attempt to explain the persistence of algal nuclear-encoded proteins in the kleptoplasts. It has been suggested that long-lived mRNA transcripts are captured along with the plastids and they are utilized as templates to produce the necessary proteins. The half-life of mRNA transcripts varies depending upon the organism and

their physiological role within the organism and is measured from minutes to days [less than a week] (Friedel *et al.*, 2009; Morey and van Dolah, 2013). It is plausible that algal mRNA transcripts are sequestered along with the plastids, but the slugs are starved for two months prior to any experimentation, so any sequestered algal mRNA from feeding would be degraded. Furthermore, it is highly unlikely that transcripts of rapidly overturned proteins could remain viable templates after almost a year of constant use (Wollman *et al.*, 1999).

The presence of cryptic algal nuclei sequestered along with the plastids has been suggested as a source of DNA, but electron micrographs of slug digestive cells have never detected the presence of algal nuclei (Graves *et al.*, 1979; Mujer *et al.*, 1996; Pierce *et al.*, 1999; Rumpho *et al.*, 2000; Mondy and Pierce, 2003). In addition, PCR control reactions to detect the presence of algal DNA do not amplify when using sea slug nucleic acid templates (Pierce *et al.*, 2007, 2009; Schwartz *et al.*, 2010)

It has been advocated that the genes required for plastid metabolism may be located within algal-derived extrachromosomal DNA that has been sequestered along with the plastids [see below] (Bhattacharya *et al.*, 2013). Extrachromosomal elements are generally 2kb to 20kb and usually consist of tandem repeats such as ribosomal DNA sequences in metazoans (Cohen *et al.*, 2008) and satellite repeat sequence units in plants (Navrátilová *et al.*, 2008). Although it is doubtful that the necessary algal nuclear genes would be located exclusively in extrachromosomal DNA, unusual coding capacity of algal cytoplasmic DNA cannot be definitively ruled out.

Finally, an unusual coding capacity of either the algal plastid or slug mitochondrial genomes was proposed to account for the genes necessary for the maintenance of the endosymbiosis. The 115.3 kb double-stranded, circular *V. litorea* plastid genome encodes ~170

genes using the standard genetic code and no algal nuclear genes were present (Rumpho *et al.*, 2008). The 14.1 kb double-stranded, circular *E. chlorotica* mitochondrial genome encodes 37 genes with unusual codon sequences for serine and leucine (serine codons: AGN and UCN, leucine codons: UAA and UAG). No algal nuclear genes were identified in the slug mitochondrial DNA (Rumpho *et al.*, 2008).

The objective of this investigation is to provide support for an alternative hypothesis for algal nuclear-encoded protein persistence in kleptoplasts; that the genes were horizontally transferred from the algal nucleus to *E. chlorotica* gDNA. First, slug and algal nucleic acids were screened for genes encoding chloroplast-targeted genes involved in pyrrole and Chla production. Algal genes targeted for potential presence in and sequencing from slug DNA included the porphyrin synthesis enzyme, *uroD*, encoding uroporphyrinogen decarboxylase, *chlD* and *chlH*, encoding subunits D and H of magnesium chelatase, the first enzyme unique to Chla synthesis, as well as *chlG*, encoding chlorophyll synthase, the final enzyme in the Chla pathway. Once detected, these genes were sequenced to make it possible to infer their original host organism prior to incorporation into the slug gDNA. Chlorophyll synthesized by the kleptoplast-*E. chlorotica* association was assayed via addition of a radiolabeled porphyrin precursor, to verify activity of the biosynthetic capabilities of the genes detected in slug gDNA.

Materials and Methods

Animals and Algae:

Elysia chlorotica were collected from a salt marsh in Menemsha on Martha's Vineyard, MA (41°21' N, 70°46' W). The animals were express shipped to our laboratory and kept at 10°C under a 14/10 light/dark cycle illuminated with cool white fluorescent bulbs in 10 gallon tanks containing aerated, filter sterilized 1000 mOsm artificial sea water (ASW: Instant Ocean). The slugs were starved for at least two months before they were used for experimentation to ensure that the gut was free from algal remnants.

Vaucheria litorea, originally collected from the same salt marsh as the slugs, was maintained in axenic culture in our laboratory. The alga was kept at 20°C under a 14/10 light/dark cycle illuminated with cool white fluorescent bulbs and maintained in modified F2 medium (Pierce *et al.*, 1996). The culture was thinned and media changed biweekly.

Elysia chlorotica do not pass plastids onto their progeny nor do the veligers ever come in contact with *V. litorea*; therefore, veligers are utilized as an algal-free nucleic acid control. Egg deposition was promoted by maintaining the animals at an elevated temperature of 22°C (Harrigan and Alkon, 1978). Egg strands were removed from the tank, rinsed with filtered 1000 mOsm ASW, placed in ASW containing 5 µg/mL rifampicin and were allowed to develop into veligers (~5 days) at RT (West, 1979). Larvae were liberated from the jelly-coat by manual shearing using a 1 mL pipet and tip. Veligers were pelleted by centrifugation at 1000 x g for 5 min at RT and the remnants of the jelly coat was removed using a 1 mL pipet. Larvae were

resuspended in ASW, poured onto a filtering apparatus fitted with a 0.45 μM screen, rinsed with ~50 mL ASW, pelleted by centrifugation and stored at -20°C until use.

Genomic DNA Purification:

Genomic DNA (gDNA) was purified from pooled batches of pre-hatched veliger larvae using the Nucleon® PhytoPure® genomic DNA extraction kit, (Tepnel Life Sciences, Manchester, UK) as per manufacturer's instructions. To purify algal gDNA filaments were rinsed with 0.2 μm filtered 250 mOsm ASW, blotted with a paper towel to remove excess moisture and crushed into a fine powder using liquid nitrogen and a mortar and pestle. The gDNA was purified using the same DNA extraction kit as the pre-hatched veliger larvae following manufacturer's instructions.

mRNA Isolation and cDNA Synthesis:

Total RNA was isolated from 2 month starved slugs by using Trizol® Reagent (Invitrogen, Carlsbad, CA) as per manufacturer instructions. The purified RNA was pelleted by centrifugation then washed twice with 75% ethanol, air dried, dissolved in RNase-free water and stored at -80°C until use. Total RNA from *V. litorea* was co-purified with its gDNA using the Nucleon® PhytoPure® genomic DNA extraction kit and was stored at -80°C until use.

Messenger RNA (mRNA) was purified from total slug or algal RNA using the Dynabeads® Oligo (dt) 25 mRNA Purification Kit (Invitrogen) following manufacturer's instructions. The purified mRNA was used immediately for cDNA synthesis. First strand cDNA was synthesized using random nonamers and PowerScript™ Reverse Transcriptase (Clontech, MountainView, CA) as per manufacturer's instructions.

Amplification and Sequencing of Chlorophyll Biosynthesis Genes:

Vaucheria litorea sequence specific oligonucleotide primers (Eurofins MWG/Operon, Huntsville, AL) were designed to amplify algal nuclear-encoded, chloroplast targeted *uroD*, *chlD*, *chlH* and *chlG* using algal transcriptome EST data (Schwartz *et al.*, 2010) (Table 1). PCR reaction mixtures contained 100 ng of gDNA or cDNA, 12.5 pmol of each primer, 0.25 mM dNTP mix (ID Labs, London, Ontario, Canada) and 1.25 units of IDProof™ DNA polymerase (ID Labs). Touchdown PCR amplifications were performed as follows: reactions were initially denatured for 2 min at 95°C, followed by 20 cycles of denaturing at 95°C for 30 s and annealing for 30 s, with the annealing temperature reduced by 1°C every other cycle, followed by an extension period of 1 min per kbp at 72°C. The samples were then denatured at 95°C for 30 s, annealed for 30 s at the lowest annealing touchdown temperature and extended at 72°C for an additional 20 cycles. The annealing temperatures for touchdown PCR started 5°C below the melting temperature of the primers.

Control reactions were tested with every PCR amplification run to ensure that algal contamination of reagents and nucleic acids were not contributing to positive reactions. A reagent control was tested using all PCR reagents except that water was replaced for the nucleic acid sample. To ensure that there was no algal contamination in gDNA or cDNA preparations, samples were amplified using primers designed to algal internal transcribed spacer 1 (*ITS1*). *ITS1* is easily amplified in organisms due to the high copy number of rRNA genes in the genome; therefore it has been used in this and previous experiments (for example Pierce *et al.*, 2007) to detect even the smallest amount of algal nuclear contamination in our nucleic acid preparations. In addition to *ITS1*, we expanded our controls to include spermidine synthase

(*SPDS*), an algal nuclear-encoded non-chloroplast targeted gene that has no function in photosynthesis or kleptoplast metabolism.

The amplicons were separated on a 1% agarose gel containing 0.2 µg/ml ethidium bromide to visualize the DNA using UV illumination, purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) and cloned using the TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. A minimum of ten clones for each gene fragment were selected and PCR amplified using M13 forward and reverse primers to ensure that the amplicon size was correct. The DNA was purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced in forward and reverse directions (Eurofins MWG/Operon).

The sequences were analyzed using the tblastx algorithm, compared to the GenBank nr database and aligned using the ClustalW2 sequence alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The gene sequences were uploaded to the NCBI GenBank database and the acquisition numbers are indicated in the results section.

Table 1: Primer Sequences for Amplification of Porphyrin and Chlorophyll Genes

<u>Gene</u>	<u>Encoded Enzyme</u>	<u>Primer Sequence</u>
<i>uroD</i>	Uroporphyrinogen decarboxylase	Forward: 5' AAAGATCCATTGTTGTTAAGG 3' Reverse: 5' GAACCTGAAGTTTTTTACGG 3'
<i>chlD</i>	Magnesium chelatase, subunit D	Forward: 5' AAAGAAATGAGCCTGAGCCG 3' Reverse: 5' GCGAAGCAATTTCAAGCATCA 3'
<i>chlH</i>	Magnesium chelatase, subunit H	Forward: 5' AGGCTTTGTATGCCAGAACC 3' Reverse: 5' ATTTTCCTCAAACCTCCCTCG 3'
<i>chlG</i>	Chlorophyll synthase	Forward: 5' TCACACCTGGAATCCATTTCGC 3' Reverse: 5' GCCATTTCTTGTATTTGGGATTT 3'

Chlorophyll a Biosynthesis:

The synthesis of Chla requires the production of tetrapyrrole compounds from the porphyrin synthesis pathway starting with the non-proteinogenic amino acid, 5-aminolevulinic acid [5-ALA] (Beale, 1999). *Elysia chlorotica* were incubated with radiolabeled-5-ALA (^{14}C -5-ALA; ^{14}C -4, 55 $\mu\text{Ci}/\text{mmol}$, American Radiolabeled Chemical, St. Louis, MO) to determine if chlorophyll production was occurring in the slug. Slugs or algal filaments (positive control) were kept under dark conditions for 8 hr then transferred to 20 mL foil-wrapped, glass scintillation vials containing either 1000 mOsm ASW/15 μCi ^{14}C -5-ALA or 250 mOsm modified F2/7.5 μCi ^{14}C -5-ALA, respectively, and incubated in the dark for 2 hr in a 25°C agitating water bath. Upon completion of the dark incubation, the foil was removed from the vials and the animals and alga were exposed to light (2-75 watt halogen flood lamps) for 18 hr in at 25°C while agitating. The slugs or algal filaments were rinsed 3x50 mL with their appropriate media to remove excess radiolabel and pigments were extracted immediately as described below. Chla can be produced either via light or dark processes. For Chla to be produced in a light-dependent fashion, protochlorophyllide reductase (POR) requires light and NADPH to catalyze the reduction of protochlorophyllide to chlorophyllide (Galova *et al.*, 2008). Therefore, to determine if the light-independent Chla pathway was used, *V. litorea* and *E. chlorotica* were incubated as described previously, except that they were kept under dark conditions.

Photosynthetic pigments including Chla were extracted from the slugs and algal filaments by homogenization in cold, HPLC grade acetone (Pinckney *et al.*, 1996). The extracts were kept on ice in foil-wrapped containers to minimize pigment damage during the extraction process. The samples were kept in the dark at -20°C until HPLC separation.

Chla was separated from other pigments and compounds utilizing HPLC (System Gold,

Beckman Coulter, Fullerton, CA) following the procedure described in Pinckney *et al.*, 1996. Two columns with unique molecular properties were run sequentially to enhance separation of the photosynthetic pigments; the high efficiency and strong retention of a monomeric C18 column (Microsorb 100-3, 100 × 4.6 mm, 3 mm, Varian, Lakeforest, CA) followed by a polymeric C18 column (Vydac 201TP, 150 × 4.6 mm, 5 mm, Vydac, Hesperia, CA) which separates similar compounds based on slight structural differences (Pinckney *et al.*, 1996). The temperature sensitive polymeric column was heated to 40°C to ensure optimal pigment separation (Van Heukelem *et al.*, 1994).

Foil-wrapped samples were kept cold and prepared for separation by the addition of ammonium acetate as an ion pair and clarified using 0.45 µm PTFE acrodisc® syringe filter (Pall® Life Sciences, Port Washington, NY). Samples were added to amber vials, placed in the autosampler and 50 µL was injected per run. A two solvent mobile phase system (solvent A: 80% MeOH:20% ammonium acetate (0.5 M, pH 7.2) and solvent B: 80% MeOH:20% acetone) was used to elute the various pigments and compounds. The separation began by using 100% Solvent A for 5 min, then changed to 50% solvent A/50% solvent B for 30 min, 100% solvent B for 8 min then finally 100% solvent A for the remainder of the run. The separation procedure was completed in 55 min and the eluting compounds were detected by UV absorbance (438 nm). Multiple chromatographic separations were performed and pooled to obtain enough material to be analyzed.

The fractions were dried using pressurized nitrogen, dissolved in 100 µL of acetone, added to scintillation cocktail (Scintisafe 30%, Fisher Scientific, Fair Lawn, NJ) and counts per minute (cpm) were determined using a liquid scintillation counter (LS6000IC, Beckman Coulter, Fullerton, CA). Acetone and Chla cause large quenching effects (Pinckney *et al.*, 1996; Borai *et*

al., 2007), so both solvent and pigment were spiked with known amounts of ^{14}C (cpm) to determine the counting efficiency. The counting efficiency was used to correct the cpm of the samples which were converted to disintegration per minute (dpm).

To confirm that Chla was radiolabeled and the detected radioactivity was not caused by a co-eluting compound, purified Chla was converted into phaeophytin (Llewellyn *et al.*, 1990; Pinckney *et al.*, 1996) and resolved via HPLC as described above. Radioactivity was also monitored to see if it shifted from Chla to the phaeophytin peak. Animals and algal filaments were incubated with ^{14}C -5-ALA and processed as described above. Samples (50 μL) were separated using HPLC utilizing the same procedure previously described and the Chla fractions from multiple runs were collected and pooled. Ten μL of 3.6 M HCl was added to the purified Chla and incubated for 5 min at RT to remove the magnesium ion from the pigment, converting it to phaeophytin. The acidified sample was neutralized with 3.6 M ammonium hydroxide, dried with nitrogen and dissolved in acetone. The sample was spiked with a known amount of unlabeled Chla to designate its elution position on the chromatograph and re-separated using HPLC. Chla and phaeophytin fractions from multiple runs were collected and pooled. Radioactivity was determined by liquid scintillation counting and the samples were corrected for quench as described above.

Results

Amplification and Sequencing of Chlorophyll Biosynthesis Genes:

Gene fragments encoding enzymes necessary for chlorophyll and porphyrin synthesis were identified in *V. litorea* and *E. chlorotica* nucleic acid preparations using PCR amplification. A single *uroD* fragment was amplified from algal cDNA (NCBI accession GU068606) and slug cDNA (GU068607). The slug *uroD* sequence differed from the algal fragment by 9 bp; eight of the differences were located in the third position without changing amino acid coding and one was found at the first position changing valine to isoleucine (Figure 1). A 957 bp fragment of *chlD* was identified in slug cDNA (GU068609) and was identical to the algal cDNA sequence (GU068608) (Figure 2). *E. chlorotica chlH* sequences amplified from slug cDNA (GU068612) and pre-hatched veliger larvae gDNA (GU068613) were 357 bp and differed by 1 bp from algal cDNA (GU068610) and gDNA (GU068611) coding regions (Figure 3) with no change to amino acid coding. A *chlG* gene fragment was identified in *E. chlorotica* cDNA (GU068615) and the 800 bp sequence was identical to *V. litorea* cDNA (GU068614) (Figure 4). No amplicons were detected in PCR reagent controls. Algal *ITS1* and *SPDS* could not be amplified from slug gDNA or cDNA.

```

V.litorea cDNA      AAAGATCCATTGTTGTTAAGGGCAGCAAGAGGAGAGGCTGTGAAAGGGTTCCTGTTTGG 60
E.chlorotica cDNA  AAAGATCCATTGTTGTTAAGGGCAGCAAGAGGAGAGGCTGTGAAAGGGTTCCTGTTTGG 60
*****

V.litorea cDNA      ATGATGAGACAGGCAGGCAGACACATGCAAGAATACAGAGACCTTCATAAAAAGTACCCC 120
E.chlorotica cDNA  ATGATGAGACAGGCAGGCAGACACATGCAAGAATACAGAGACCTTCATAAAAAGTACCCC 120
*****

V.litorea cDNA      ACATTCAGAGAGAGATCGGAGATCCACGAAGTGTCCACTGAAATTTCACTTCAGCCTTAC 180
E.chlorotica cDNA  ACATTCAGAGAGAGATCGGAGATCCACGAAGTGTCCACTGAAATTTCACTTCAGCCTTAC 180
*****

V.litorea cDNA      AGGCGATAAGGAACCGATGGAGTGATCTTATTTTCTGATATTCGACTCCACTGCCTGGA 240
E.chlorotica cDNA  AGGCGATAAGGAACCGATGGAGTGATCTTATTTTCTGATATTCGACTCCACTGCCTGGA 240
*****

V.litorea cDNA      ATGGGTGTCGATTTCAAATGAAGAGAAAACCGGGCCAAATGGTCCCAATGAGAAC 300
E.chlorotica cDNA  ATGGGTGTCGATTTCAAATGAAGAGAAAACCGGGCCAAATGGTCCCAATGAGAAC 300
*****

V.litorea cDNA      TGGGAAAGTGTCAATGCAATGCACACAATTGATTCTGAAAAGGCATGTCCTTTGTGGGG 360
E.chlorotica cDNA  TGGGAAAGTGTCAATGCAATGCACACAATTGATTCTGAAAAGGCATGTCCTTTGTGGGG 360
*****

V.litorea cDNA      CAAACTCTGAGAGATTTGAAAAAGAGGTCGGATCAAATGCAACAGTCCTGGGTTTTGTG 420
E.chlorotica cDNA  CAAACTCTGAGAGATTTGAAAAAGAGGTCGGATCAAATGCAACAGTCCTGGGTTTTGTG 420
*****

V.litorea cDNA      GGATGTCGGTACACACTTGCACCTTACATGGTGAAGGAGGTTCAAGCAGAGAATATTG 480
E.chlorotica cDNA  GGATGTCGGTACACACTTGCACCTTACATGGTGAAGGAGGTTCAAGCAGAGAATATTG 480
*****

V.litorea cDNA      GAAATTAAGAAGATGATGTTCACTGAGCCTGAGTTGTTGCATGCCATGCTGGCCAAAAT 540
E.chlorotica cDNA  GAAATTAAGAAGATGATGTTCACTGAGCCTGAGTTGTTGCATGCCATGCTGGCCAAAAT 540
*****

V.litorea cDNA      GCTGATTCAATAGGGGATTATGGGATTATCAAATCGAAAGTGGTGCACAGGTGATTCAA 600
E.chlorotica cDNA  GCTGATTCAATAGGGGATTATGGGATTATCAAATCGAAAGTGGTGCACAGGTGATTCAA 600
*****

V.litorea cDNA      GTCTTTGACTCTTGGGCAGGCCATCTTCCCCCAAAGACTATGATGTTTTGCGGCACCT 660
E.chlorotica cDNA  GTCTTTGACTCTTGGGCAGGCCATCTTCCCCCAAAGACTATGATGTTTTGCGGCACCT 660
*****

V.litorea cDNA      TACCAAAGAAGGTTATCCAAAAATCAAATCTTCTCATCCTGAAGTCCCATCATCATT 720
E.chlorotica cDNA  TACCAAAGAAGGTTATCCAAAAATCAAATCTTCTCATCCTGAAGTCCCATCATCATT 720
*****

V.litorea cDNA      TACATAAACAGAGTGGTGCACCTTTGGAAAGGATGAGTCAAAGTGGGGCAGATATCATC 780
E.chlorotica cDNA  TACATAAACAGAGTGGTGCACCTTTGGAAAGGATGAGTCAAAGTGGGGCAGATATCATC 780
*****

V.litorea cDNA      AGCTTGGATTGGACAGTGACGATTGAAGAGGCTAGGAAAAGAATGGCAACGATATTGGC 840
E.chlorotica cDNA  AGCTTGGATTGGACAGTGACGATTGAAGAGGCTAGGAAAAGAATGGCAACGATATTGGC 840
*****

V.litorea cDNA      ATCCAGGGTAACCTTGATCCAGCTGCCTTGTGTCACCAATGAAGTTATCAAGGAAAGG 900
E.chlorotica cDNA  ATCCAGGGTAACCTTGATCCAGCTGCCTTGTGTCACCAATGAAGTTATCAAGGAAAGG 900
*****

V.litorea cDNA      ACTGAAGAAATTTGAGGGCATGCGGAGGGGAGAAACCATGTCATGAATTTGGGCCATGGA 960
E.chlorotica cDNA  ACTGAAGAAATTTGAGGGCATGCGGAGGGGAGAAACCATGTCATGAATTTGGGCCATGGA 960
*****

V.litorea cDNA      ATCGAAGCGACGACTTCAGAAGAAAAGGCTGAATTTTTCATCAATACCGTAAAAAACTTC 1020
E.chlorotica cDNA  ATCGAAGCGACGACTTCAGAAGAAAAGGCTGAATTTTTCATCAATACCGTAAAAAACTTC 1020
*****

V.litorea cDNA      AGGTTT 1026
E.chlorotica cDNA  AGGTTT 1026
*****

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Figure 1*: Comparison of *Vaucheria litorea* and *Elysia chlorotica* Uroporphyrinogen Decarboxylase (*uroD*) cDNA sequences. Comparison of consensus nucleotide alignments of *uroD* located in the transcriptome data and cDNA of *V. litorea* with that found by PCR in *E. chlorotica* cDNA. The two sequences are extremely similar in composition over the 1026 bases differing by 9 bases (■). The slug sequences come from at least 3 separate mRNA extractions done on at least 3 different groups of slugs at different times of the year.*Figure from Pierce *et al.*, 2009

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V.litorea cDNA      AAAGAAATGAGCCTGAGCCGAAAATCAGCCCAGAGATGACGAAGCCCCATCTGTACCCC 60
E.chlorotica cDNA  AAAGAAATGAGCCTGAGCCGAAAATCAGCCCAGAGATGACGAAGCCCCATCTGTACCCC 60
*****

V.litorea cDNA      AAGAATTCATGTTTGGCATCGATTCAACGGTCATCGACCCTGAACTGTTGGATTTCCGGAC 120
E.chlorotica cDNA  AAGAATTCATGTTTGGCATCGATTCAACGGTCATCGACCCTGAACTGTTGGATTTCCGGAC 120
*****

V.litorea cDNA      GGAAGAACAATGCCGGCAGGCTCTGGGAAGAGGGGAATGATCTTTAACATGGAAGAGGGC 180
E.chlorotica cDNA  GGAAGAACAATGCCGGCAGGCTCTGGGAAGAGGGGAATGATCTTTAACATGGAAGAGGGC 180
*****

V.litorea cDNA      GATATATCAAGCCGATGCTTCCGAAAGGAAAAAAGGAAATTTGGCGTTGGATGCGACGC 240
E.chlorotica cDNA  GATATATCAAGCCGATGCTTCCGAAAGGAAAAAAGGAAATTTGGCGTTGGATGCGACGC 240
*****

V.litorea cDNA      TGAGATCAGCGCGCCGTATCAATTGTGCGAGAAGATCGCGCGCTCTCTCAAAGAACGACG 300
E.chlorotica cDNA  TGAGATCAGCGCGCCGTATCAATTGTGCGAGAAGATCGCGCGCTCTCTCAAAGAACGACG 300
*****

V.litorea cDNA      GGAATCCGACAAAAGAACAGTCTTTGTGCAAAAAGTCTGATCTGAGGGTCAAAAAGCTCG 360
E.chlorotica cDNA  GGAATCCGACAAAAGAACAGTCTTTGTGCAAAAAGTCTGATCTGAGGGTCAAAAAGCTCG 360
*****

V.litorea cDNA      CGCGGAAAGCCGGCTCACTTATCATTTTCTGCGTTGACGCGAGCGGGAGCATGGCGCTGA 420
E.chlorotica cDNA  CGCGGAAAGCCGGCTCACTTATCATTTTCTGCGTTGACGCGAGCGGGAGCATGGCGCTGA 420
*****

V.litorea cDNA      ACCGAATGAACGCCGCGAAAAGGCGCAGCAATGTCATTGCTGGGCGAAGCCTACAAAAGCA 480
E.chlorotica cDNA  ACCGAATGAACGCCGCGAAAAGGCGCAGCAATGTCATTGCTGGGCGAAGCCTACAAAAGCA 480
*****

V.litorea cDNA      GGGACAAAGTGTGCCTCATACTTTCCAGGGGAAAGGGCTGAAGTCCTCCTCCCACCTT 540
E.chlorotica cDNA  GGGACAAAGTGTGCCTCATACTTTCCAGGGGAAAGGGCTGAAGTCCTCCTCCCACCTT 540
*****

V.litorea cDNA      CAAGTTCAATAGCAATGGCAAAAAGCCGTTTGGAGACGATGCCGTGTGGAGGTGGGTAC 600
E.chlorotica cDNA  CAAGTTCAATAGCAATGGCAAAAAGCCGTTTGGAGACGATGCCGTGTGGAGGTGGGTAC 600
*****

V.litorea cDNA      CGCTCGCTCATGCAATCAACGTCGCTGTACGGACAGGGATTAACGCCATCAAATCACAGG 660
E.chlorotica cDNA  CGCTCGCTCATGCAATCAACGTCGCTGTACGGACAGGGATTAACGCCATCAAATCACAGG 660
*****

V.litorea cDNA      ACGTGGGAAAAGTGGTGATTTGTGATGGTAAGCGATGGTCGAGCGAATGTGCCCTCGCGG 720
E.chlorotica cDNA  ACGTGGGAAAAGTGGTGATTTGTGATGGTAAGCGATGGTCGAGCGAATGTGCCCTCGCGG 720
*****

V.litorea cDNA      TCAGTAACGGCACGCAGCTCCCTGAAGATGAGAAGATGTCCAGGGAGGAGTTGAAGGAGG 780
E.chlorotica cDNA  TCAGTAACGGCACGCAGCTCCCTGAAGATGAGAAGATGTCCAGGGAGGAGTTGAAGGAGG 780
*****

V.litorea cDNA      AGGTGTTGAACACTGCGAAGGCGCTGAGGGAGTTGCCGGCCTTTAGCTTGGTGGTGTGG 840
E.chlorotica cDNA  AGGTGTTGAACACTGCGAAGGCGCTGAGGGAGTTGCCGGCCTTTAGCTTGGTGGTGTGG 840
*****

V.litorea cDNA      ACACTGAGAATAAGTTCGTGAGCACTGGCATGGCGAAGGAGTTGGCTGCAGCGGCTGGTG 900
E.chlorotica cDNA  ACACTGAGAATAAGTTCGTGAGCACTGGCATGGCGAAGGAGTTGGCTGCAGCGGCTGGTG 900
*****

V.litorea cDNA      GGAGATATCATTATATTTCCAAAAGCAACGGATCAAGCGATGGCGAAGGTGGCCAGCGAAG 960
E.chlorotica cDNA  GGAGATATCATTATATTTCCAAAAGCAACGGATCAAGCGATGGCGAAGGTGGCCAGCGAAG 960
*****

V.litorea cDNA      CAATTTCAAGCATCA 975
E.chlorotica cDNA  CAATTTCAAGCATCA 975
*****

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Figure 2*: Comparison of *Vaucheria litorea* and *Elysia chlorotica* magnesium chelatase, subunit D (*chlD*) cDNA sequences. Nucleotide alignment of consensus *chlD* sequences located by PCR in *V. litorea* and *E. chlorotica* cDNA using primer sequences based on the *V. litorea* transcriptome data. These gene fragments match exactly over the 975 bases. The *E. chlorotica* data come from 3 separate mRNA extractions done on at least 3 different groups of slugs at different times of the year.

*Figure from Pierce *et al.*, 2009

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V.litorea cDNA      AGGCTTTGTATGCCAGAACCAAACCTCTTGAACCCGAAGTTCTACGAGGGG 50
V.litorea gDNA     AGGCTTTGTATGCCAGAACCAAACCTCTTGAACCCGAAGTTCTACGAGGGG 50
E.chlorotica cDNA  AGGCTTTGTATGCCAGAACCAAACCTCTTGAACCCGAAGTTCTACGAGGGG 50
E.chlorotica veliger gDNA  AGGCTTTGTATGCCAGAACCAAACCTCTTGAACCCGAAGTTCTACGAGGGG 50
*****

V.litorea cDNA     ATGTTGAACAGTGGGTACGAGGGCACAAGGGAGATCACCAAAGGCTGAG 100
V.litorea gDNA     ATGTTGAACAGTGGGTACGAGGGCACAAGGGAGATCACCAAAGGCTGAG 100
E.chlorotica cDNA  ATGTTGAACAGTGGGTACGAGGGCACAAGGGAGATCACCAAAGGCTGAG 100
E.chlorotica veliger gDNA  ATGTTGAACAGTGGGTACGAGGGCACAAGGGAGATCACCAAAGGCTGAG 100
*****

V.litorea cDNA     AAATACCATGGGATGGTCTGCCACTGCAGGGGAGGTGGACAACCTTTATCT 150
V.litorea gDNA     AAATACCATGGGATGGTCTGCCACTGCAGGGGAGGTGGACAACCTTTATCT 150
E.chlorotica cDNA  AAATACCATGGGATGGTCTGCCACTGCAGGGGAGGTGGACAACCTTTATCT 150
E.chlorotica veliger gDNA  AAATACCATGGGATGGTCTGCCACTGCAGGGGAGGTGGACAACCTTTATCT 150
*****

V.litorea cDNA     ACGAAGATGCGAACGATGTTTCATCAAAGATGAAGCCATGAGGGAGAGA 200
V.litorea gDNA     ACGAAGATGCGAACGATGTTTCATCAAAGATGAAGCCATGAGGGAGAGA 200
E.chlorotica cDNA  ACGAAGATGCGAACGATGTTTCATCAAAGATGAAGCCATGAGGGAGAGA 200
E.chlorotica veliger gDNA  ACGAAGATGCGAACGATGTTTCATCAAAGATGAAGCCATGAGGGAGAGA 200
*****

V.litorea cDNA     CTGCTCAATACCAATCCGAACGCCTTCCGCGACATGATCACCACCTTTTCT 250
V.litorea gDNA     CTGCTCAATACCAATCCGAACGCCTTCCGCGACATGATCACCACCTTTTCT 250
E.chlorotica cDNA  CTGCTCAATACCAATCCGAACGCCTTCCGCGACATGATCACCACCTTTTCT 250
E.chlorotica veliger gDNA  CTGCTCAATACCAATCCGAACGCCTTCCGCGACATGATCACCACCTTTTCT 250
*****

V.litorea cDNA     GGAGGCCAATGGAAGGGGCTACTGGGACACCTCGGATGATAATATAGAAC 300
V.litorea gDNA     GGAGGCCAATGGAAGGGGCTACTGGGACACCTCGGATGATAATATAGAAC 300
E.chlorotica cDNA  GGAGGCCAATGGAAGGGGCTACTGGGACACCTCGGATGATAATATAGAAC 300
E.chlorotica veliger gDNA  GGAGGCCAATGGAAGGGGCTACTGGGACACCTCGGATGATAATATAGAAC 300
*****

V.litorea cDNA     TGTTCAGGATCTGTACCAAGAGGTGGAAGATAAAAATCGAGGGAGTTTGA 350
V.litorea gDNA     TGTTCAGGATCTGTACCAAGAGGTGGAAGATAAAAATCGAGGGAGTTTGA 350
E.chlorotica cDNA  TGTTCAGGATCTGTACCAAGAGGTGGAAGATAAAAATCGAGGGAGTTTGA 350
E.chlorotica veliger gDNA  TGTTCAGGATCTGTACCAAGAGGTGGAAGATAAAAATCGAGGGAGTTTGA 350
*****

V.litorea cDNA     GGAAAAT 357
V.litorea gDNA     GGAAAAT 357
E.chlorotica cDNA  GGAAAAT 357
E.chlorotica veliger gDNA  GGAAAAT 357
*****

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Figure 3*: Comparison of *Vaucheria litorea* and *Elysia chlorotica* magnesium chelatase, subunit H (*chlH*) cDNA and gDNA sequences. Nucleotide alignment of consensus sequences of *chlH* in cDNA and genomic DNA from *V. litorea* and also cDNA from *E. chlorotica* adults and genomic DNA from pre-hatched *E. chlorotica* veliger larvae. The sequences differ in 1 nucleotide between algae and slug (■). The sequence data were the same among at least 3 different DNA or mRNA extractions from at least 3 different groups of organisms at different times of the year.

* Figure from Pierce *et al.*, 2009

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V.litorea cDNA      TCACACCTGGAATCCATTTCGACGGGCCAGATGCAGTTGATTTACAAGATGCTGGGATTGA 60
E.chlorotica cDNA  TCACACCTGGAATCCATTTCGACGGGCCAGATGCAGTTGATTTACAAGATGCTGGGATTGA 60
*****

V.litorea cDNA      CTGGCCAAAGCTCTGACTTGTATGATATTTGGCTGGGCCCTTCTAACTGGCTTTACCCA 120
E.chlorotica cDNA  CTGGCCAAAGCTCTGACTTGTATGATATTTGGCTGGGCCCTTCTAACTGGCTTTACCCA 120
*****

V.litorea cDNA      AACCATCAACGATTGGTATGACCGAGATATTGATGCGATCAATGAGCCATATCGACCCAT 180
E.chlorotica cDNA  AACCATCAACGATTGGTATGACCGAGATATTGATGCGATCAATGAGCCATATCGACCCAT 180
*****

V.litorea cDNA      TCCTTCTGGAGCTATTTCTGAGGGTCAAGTGAAAGCGCAAATTCGCTTTCTCTAGTTGG 240
E.chlorotica cDNA  TCCTTCTGGAGCTATTTCTGAGGGTCAAGTGAAAGCGCAAATTCGCTTTCTCTAGTTGG 240
*****

V.litorea cDNA      TGGATTGGCTTTGTTCGTATGGTTTGGATCTATGGGCAGGGCACCAAATGCCCACTGTTTT 300
E.chlorotica cDNA  TGGATTGGCTTTGTTCGTATGGTTTGGATCTATGGGCAGGGCACCAAATGCCCACTGTTTT 300
*****

V.litorea cDNA      TTTGTTGTCATTGTTTGGGACTTTCATTTTCATACATATACTCAGCCCCGCCACTGAAATT 360
E.chlorotica cDNA  TTTGTTGTCATTGTTTGGGACTTTCATTTTCATACATATACTCAGCCCCGCCACTGAAATT 360
*****

V.litorea cDNA      GAAACAGAATGGCTGGGCAGGTAATTTTGCCTTGGGCTCAAGCTACATTAGCTTGCCGTG 420
E.chlorotica cDNA  GAAACAGAATGGCTGGGCAGGTAATTTTGCCTTGGGCTCAAGCTACATTAGCTTGCCGTG 420
*****

V.litorea cDNA      GTGGTGTGGTCAGGCTATGTTTGGTGAGCTCAACTTGCAAGTGTGGTCTTAACCTTTGCT 480
E.chlorotica cDNA  GTGGTGTGGTCAGGCTATGTTTGGTGAGCTCAACTTGCAAGTGTGGTCTTAACCTTTGCT 480
*****

V.litorea cDNA      GTATTCTTGGGCAGGCCTTGGAAATGCAATAGTAAATGACTTCAAATCAGTTGAGGGGGA 540
E.chlorotica cDNA  GTATTCTTGGGCAGGCCTTGGAAATGCAATAGTAAATGACTTCAAATCAGTTGAGGGGGA 540
*****

V.litorea cDNA      TAGAGCCATGGGTTTACAGTCTCTTCCGTGGCTTTTGGTATAGAAAAGCCAAGTGGAT 600
E.chlorotica cDNA  TAGAGCCATGGGTTTACAGTCTCTTCCGTGGCTTTTGGTATAGAAAAGCCAAGTGGAT 600
*****

V.litorea cDNA      ATGTGTGAGTAGCATTGACATTACTCAATTGGGCATAGCCGCATGGCTATATTATATTGG 660
E.chlorotica cDNA  ATGTGTGAGTAGCATTGACATTACTCAATTGGGCATAGCCGCATGGCTATATTATATTGG 660
*****

V.litorea cDNA      AGAGCCTACCTATGCATTTCGTTTTATTGGGCCTCATTCTTCCCTCAGATATATGCACAATT 720
E.chlorotica cDNA  AGAGCCTACCTATGCATTTCGTTTTATTGGGCCTCATTCTTCCCTCAGATATATGCACAATT 720
*****

V.litorea cDNA      TAAGTATTTTTTGCCGGATCCAGTTGAGAATGATGTCAAATACCAAGGATTTGCTCAGCC 780
E.chlorotica cDNA  TAAGTATTTTTTGCCGGATCCAGTTGAGAATGATGTCAAATACCAAGGATTTGCTCAGCC 780
*****

V.litorea cDNA      ATTTCTTGATTTGGGATTT 800
E.chlorotica cDNA  ATTTCTTGATTTGGGATTT 800
*****

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Figure 4*: Comparison of *Vaucheria litorea* and *Elysia chlorotica* chlorophyll synthase (*chlG*) cDNA sequences. Nucleotide alignments of consensus sequences for *chlG*, the terminal enzyme in the Chl_a synthesis pathway, identified in cDNA from *V. litorea* and *E. chlorotica*. The sequences match 100% over the 800base run. As with the preceding figures, these fragments were produced by PCR using primer sequences made from the *V. litorea* transcriptome data. The *E. chlorotica* data come from 3 separate mRNA extractions done on at least 3 different groups of slugs at different times of the.

*Figure from Pierce *et al.*, 2009.

Chlorophyll a Biosynthesis:

Acetone extracts of *V. litorea* and *E. chlorotica* were resolved using HPLC and the chromatograms were compared. The localization of the Chla peak was determined by the use of a standard. A number of smaller peaks were observed during the algal extract separation, but a large peak was detected at ~43.5 min which corresponded to the same location as the Chla standard peak on the chromatogram. The radioactivity measured from fractions collected from the algal extract consisted of a large void volume peak from 4-8 min, a single peak at ~20 min, a number of peaks from ~40-43 and a single large peak at ~43.5 min (Figure 5a). The chromatogram of the slug extract looked very similar to the algal extract separation with the major peak detected at ~43.5 min corresponding to Chla, with a void volume peak (4-8 min). Additional peaks were also apparent from slug extract: a large peak from ~12-16 min and a number of smaller peaks from ~16-43 min and ~45-50 min (Figure 5b).

Verifying that Chla was indeed radiolabeled, peaks from 43.5 min, when acid-treated, shifted to ~56 min, consistent with conversion to phaeophytin (Figure 6). Further, extracts from slugs incubated in the dark did not have a radiolabeled peak at 43.5 min (Figure 7).

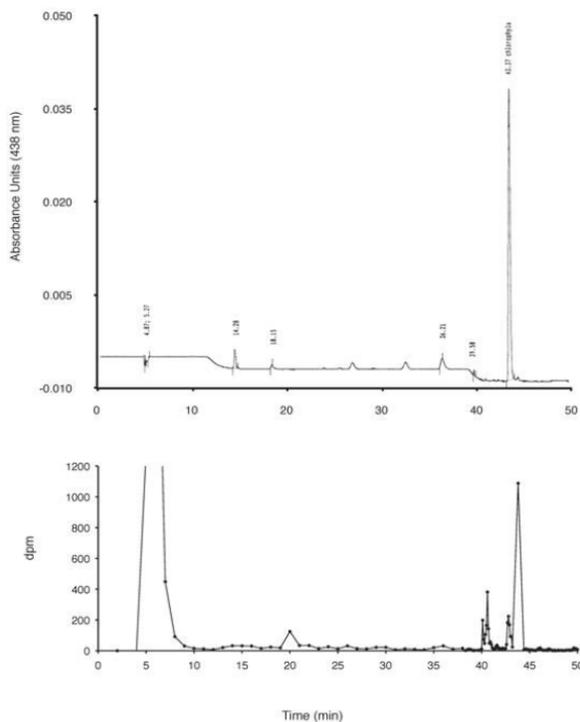


Figure 5a*: *Vaucheria litorea* Photosynthetic Pigment Separation and Corresponding Radioactivity (dpm) of Fractions. Typical HPLC chromatogram of Chla extracted from *V. litorea* (upper chart) and separated according to the protocol described in the methods section. The Chla peak is labeled such at approximately 43.5 min. The lower chart represents the radioactivity (^{14}C) in fractions collected from the HPLC column eluant also as described in the methods. The large peak in radioactivity at approximately 43.5 min coelutes exactly with the Chla peak in the upper chart. Although we did not identify them, the smaller radioactive peaks just preceding the Chla location are most likely intermediates in the Chla synthesis pathway (see Pinckney *et al.*, 1996). The large peak of radioactivity starting at about 4 min, which was not detected on the HPLC chromatogram, is right at the column void volume.

*Figure from Pierce *et al.*, 2009

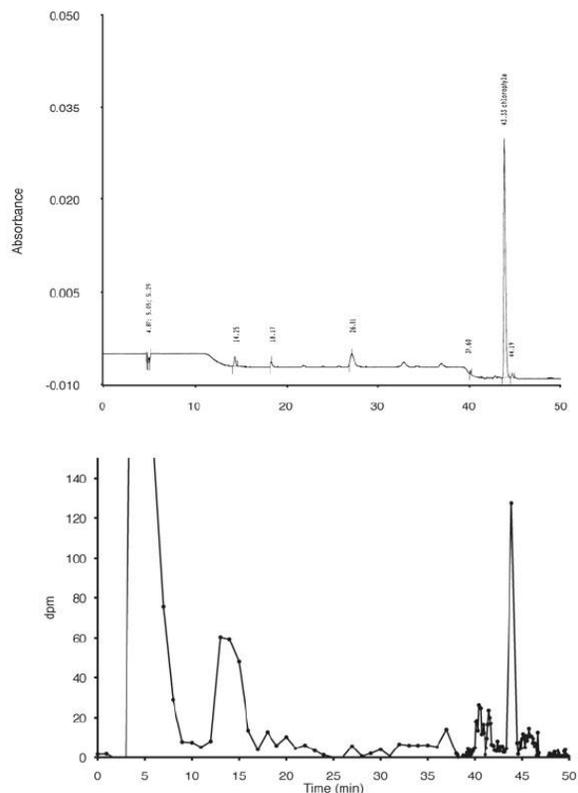


Figure 5b*: *Elysia chlorotica* Photosynthetic Pigment Separation and Corresponding Radioactivity (dpm) of Fractions. Typical HPLC chromatogram (upper chart) of Chla extracted from *E. chlorotica* and separated by the same protocol that produced the *V. litorea* results in Fig.5a. The peak at approximately 43.5 min corresponds to the elution time of both standard Chla (Sigma Chemicals) as well as the Chla peak in the *V. litorea* chromatogram. The lower chart represents the (^{14}C) radioactivity profile in fractions of eluant collected during the HPLC run. The large peak of radioactivity at approximately 43.5 min corresponds exactly with the elution of the Chla peak. The large peak starting at about 4 min into the run is right at the column void volume. The identities of the other radioactive peaks are unknown. Those just preceding and following Chla are likely Chla precursors and degradation products (Llewellyn *et al.*, 1990; Pinckney *et al.*, 1996; Nayar *et al.*, 2003). The broad peak from 13–15 min is in the region where chlorophyll c and fucoxanthin elute in this HPLC protocol (Llewellyn *et al.*, 1990).

*Figure from Pierce *et al.*, 2009

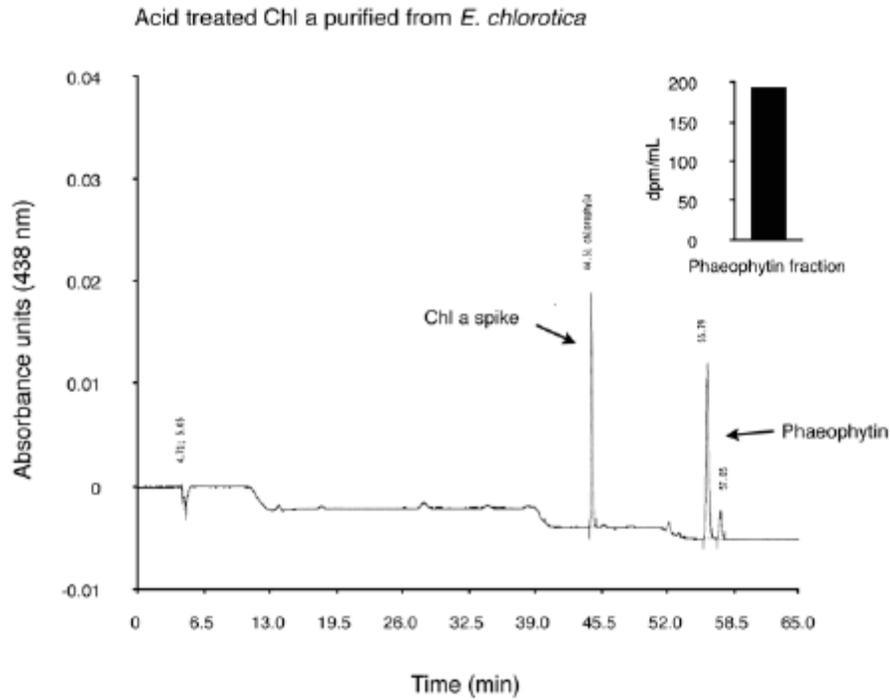


Figure 6*: Acid treated Chlorophyll a Purified from *Elysia chlorotica*. A typical chromatogram showing the results of conversion of radioactive chlorophyll purified from *E. chlorotica* to phaeophytin by acid treatment as described in the methods. Initially, radioactive Chla was collected as usual from the HPLC. Neither peak nor radioactivity was recovered in the region where phaeophytin elutes (55.8 min). The collected Chla was acid treated as described; the extract was spiked with non-radioactive Chla to mark its elution point (arrow) and rechromatographed. As shown here, a new radioactive peak (inset) has appeared which co-elutes with phaeophytin (Llewellyn *et al.*, 1990) (arrow) and is well separated from the Chla spike.

*Figure from Pierce *et al.*, 2009

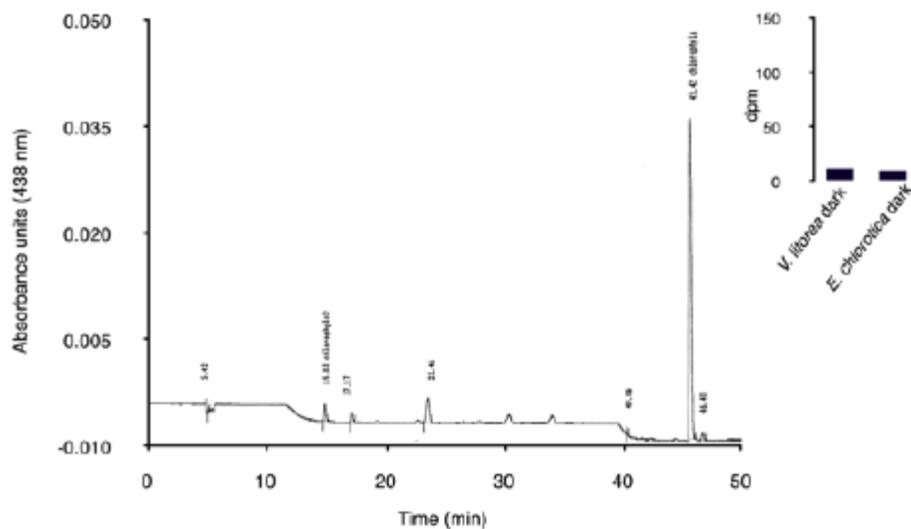


Figure 7*: *Elysia chlorotica* Photosynthetic Pigment Separation from Dark Exposure Conditions. Typical HPLC chromatogram of Chla and associated radioactivity following incubation of slugs with ^{14}C -ALA in the dark and extraction as described in the methods. As in the other figures, Chla is the peak at 45 min labeled “chlorophyll a”. The histogram inset displays the small amount of radioactivity that was incorporated in Chla by slugs and algal filaments during an 18 hr dark incubation, indicating that almost no Chla synthesis occurs in either the algal filaments or the symbiotic chloroplasts without the presence of light (compare to Figs. 5a and b).

*Figure from Pierce *et al.*, 2009

Discussion

For ongoing photosynthetic activity to occur in plants and algae, replacement of photo damaged Chla is necessary; therefore the identification of genes involved in porphyrin (*uroD*) and chlorophyll (*chlD*, *chlH* and *chlG*) production in *E. chlorotica* nucleic acids as well as *in vivo* radiolabeling of Chla strongly suggests that the slug is manufacturing chlorophyll to replace the photosynthetic pigment damaged during light capture. The shifting of radioactivity from acid-treated Chla to phaeophytin indicates that the pigment is radiolabeled and the activity detected is not attributed to a co-eluting compound. The production of Chla by the kleptoplasts utilizing proteins encoded by both slug nuclear DNA and the plastid genome provides the first biochemical evidence supporting the horizontal transfer of functional *V. litorea* nuclear genes, possibly for the complete pyrrole and Chla biosynthesis pathways, to the *E. chlorotica* genome.

One possible alternate explanation for Chla production by kleptoplasts is that this pigment is synthesized using enzymes for light-independent Chla production, encoded by genes in the *V. litorea* plastids. However, the lack of radiolabeled pigment in both the slug and alga during dark exposure indicates that the light-dependent Chla pathway is solely utilized under our experimental conditions. Since ¹⁴C-5-ALA was added after an 8 hr dark period, it is likely that light-independent Chla production would have either concluded or that sufficient amounts of pigment are produced using the light-dependent pathway.

Another possibility is that chlorophyll biosynthesis is facilitated by slug native porphyrin synthesis enzymes. In animals, heme production occurs partially in the cytoplasm (from 5-ALA

to coproporphyrinogen III) and the remaining steps transpire in the mitochondria (Thunell, 2000), but in photosynthetic organisms tetrapyrrole, chlorophyll and heme production proceeds in the chloroplast envelope, stroma and/or thylakoids using a combination of nuclear and plastid-encoded enzymes (Tanaka and Tanaka, 2006). Therefore, it would be unlikely that native slug enzymes could be interchanged for algal nuclear derived proteins, since they do not possess a chloroplast targeting signal peptide. The identification of an algal derived *uroD* fragment in slug cDNA suggests that chloroplast-targeted enzymes are indeed necessary for porphyrin production within the plastid.

Independent operation of two porphyrin biosynthetic pathways in the slug is also suggested by expressed sequence tag data from *E. chlorotica*, which includes a native *uroD* sequence (GU559722) (Schwartz *et al.*, 2010). The amino acid sequence of this native *uroD* is only 27% identical to the algal gene, but 84% identical to *uroD* from the non-plastid sequestering sacoglossan, *A. californica* (NCBI accession # XP_005105887). The algal *uroD* sequence encoded by the slug genome is nearly 100% identical to the amino acid region of the *V. litorea* fragment. The presence of innate and algal derived *uroD* transcripts encoding enzymes with divergent amino acid sequences for reactions that proceed in different cellular compartments provides evidence that these enzymes are not used interchangeably for producing tetrapyrrole compounds. Although these data strongly suggest that algal derived enzymes are solely used for tetrapyrrole production within the kleptoplast, further experimentation must be completed to identify the remaining genes in the porphyrin synthesis pathway.

The amplification of the Chla pathway gene fragments, *chlD*, *chlH* and *chlG* from *E. chlorotica* nucleic acids confirms the presence of pigment production enzyme templates within the slug genome. The identification of all three genes in cDNA verifies the transcription of

algal-derived nuclear DNA using *E. chlorotica*'s cellular machinery and the presence of *chlH* in pre-hatched veliger larvae gDNA indicates that the algal nuclear gene has been horizontally transferred to the slug genome. The presence of *chlD* and *chlG* has also been confirmed in pre-hatched veliger gDNA (Schwartz *et al.*, 2010). An 899 bp *chlD* (GU559724) fragment was amplified and is 100% identical to algal gDNA exon and intron regions. A 233 bp *chlG* (GU559726) fragment was identified and is also 100% identical to algal gDNA exon regions, but does not contain an intron that is present within the algal sequence.

Nearly one dozen algal nuclear-encoded genes have been identified in slug nucleic acid preparations using PCR amplification (Pierce *et al.*, 2007, 2009; Rumpho *et al.*, 2008, 2009; Schwartz *et al.*, 2010) and many of the nucleotide sequences are 100% identical within exon and intron regions. The sequence identity indicates that these genes may have been transferred recently in geological time; therefore not having enough time to mutate and change coding regions. When nucleotides differ between algal and slug sequences it is usually only one residue located in the third position without a change in coding. This minimal change also indicates a more recent transfer, but a different transfer event at a slightly earlier time period. The identification of the algal *uroD* sequence that had significant differences within the coding region was the first time that more than a slight variation between algal and slug sequences was observed. Most of the differences were located in the third position without change in amino acid coding and one difference occurred in the first position changing valine to isoleucine (silent substitution). The residue differences may be due to a transfer event that occurred earlier in time resulting in third position changes corresponding to the organism's coding preference (Kurlan, 1991) or it may be due to clinal variation within closely related populations of *V. litorea* (Schmidt *et al.*, 2008). The absence of intron sequences in genes amplified from pre-hatched

veliger larval gDNA has been observed in *chlG*, *prk*, encoding the Calvin-Benson cycle enzyme phosphoribulokinase, and *Lhcv-3*, encoding light-harvesting complex protein 3 (Schwartz *et al.*, 2010). These intron-free sequences may be the products of retroviral insertion of reverse-transcribed mRNA sequences within the slug germline (see below).

There has been concern that algal contamination of slug nucleic acid preparations may result in false positive reactions due to the sensitivity of PCR amplification; however, attempts to amplify other algal nuclear-encoded genes (*ITS1* and *SPDS*) were unsuccessful. Further, when algal nuclear genes were initially identified using PCR amplification, a laboratory control was performed where all new reagents and disposables were shipped to a laboratory that had never been in contact with *V. litorea* to verify that the positive results were not due to algal contamination of our laboratory. Slug gDNA was extracted and amplified in the new laboratory resulting in negative reagent and *ITS1* controls and an amplicon for a previously identified algal gene, *fcp*, encoding fucoxanthin chlorophyll *a/b* binding protein, which was verified by DNA sequencing (Pierce *et al.*, 2007). Collectively, these controls strongly indicate that algal contamination of our nucleic acid preparations is not the cause for positive results.

This laboratory has proposed that the *E. chlorotica* digestive cell/*V. litorea* plastid association is maintained by the expression of genes that have been transferred from the algal nucleus. Along with previous molecular evidence supporting HGT (Pierce *et al.*, 2007, 2009, 2012; Rumpho *et al.*, 2008, 2009; Schwartz *et al.*, 2010) the identification of algal porphyrin and Chla biosynthesis genes in pre-hatched veliger larvae gDNA and *de novo* Chla production by the kleptoplast suggests that the genes have been successfully incorporated into the slug genome, are capable of encoding biologically functional, chloroplast-targeted proteins (ie. transcription and translation) and are vertically transmitted to their progeny.

Genomic sequencing of *E. chlorotica* egg gDNA, a source of plastid-free nucleic acids, detected no transferred algal genes in the slug genome (Bhattacharya *et al.*, 2013). This finding (or in this case, lack of finding) lead the authors to conclude that HGT plays little to no role in the maintenance of the slug/algal endosymbiotic relationship and that another entity, such as extrachromosomal DNA obtained from the alga when feeding, may may be responsible for maintaining the association. Slug larval DNA sequencing efforts have been attempted by our laboratory in conjunction with our collaborators at the Beijing Genomics Institute (BGI) who successfully sequenced the *E. chlorotica* transcriptome and the *V. litorea* genome and transcriptome (Pierce *et al.*, 2012). Sequence assembly proved to be nearly impossible because of sample heterozygosity, due to pooling of DNA extracted from hundreds of egg masses to obtain the large amount of nucleic acids required for sequencing. Therefore, questions can be raised concerning contig assembly in the aforementioned study for similar reasons. Raw slug reads were also compared to the algal transcriptome and no transferred algal genes were identified. This evidence suggests that HGT may not have occurred or that if it did, it was not due to a large scale gene transfer event. A viable alternate explanation would be that the slug genome was not fully sequenced. For example, *prk* was recently localized on an *E. chlorotica* metaphase chromosome indicating that HGT has occurred from alga to slug (Schwartz *et al.*, 2014), but this sequence was not identified in the slug genome. In addition, extrachromosomal DNA is co-purified with gDNA in plants (Navrátilová *et al.*, 2008), so if algal cytoplasmic DNA with an usual coding capacity is partially responsible for the algal nuclear gene templates in slug larval DNA preparations, they have not been identified in this genome data set either. Although the *E. chlorotica* genome size has yet to be determined, the non-plastid sequestering sacoglossan sea hare, *A. californica*, has recently been sequenced by the Broad Institute of Harvard and MIT

and the genome was found to be 712 Mbp. Given the fragmented nature of the slug egg gDNA sequences and the large size of a related sacoglossan genome, it may be possible that larger sequencing efforts using newer technology could generate longer ESTs to be used to facilitate better assembly of the slug genome. In addition, advances in single cell DNA sequencing are rapidly occurring (Macaulay and Voet, 2014), so this may currently be the best approach to eradicate heterozygosity issues to facilitate the assembly of deep sequencing efforts. It is presently unclear if the gene transfer entails large pieces of DNA, single genes, or multiple transfer events incorporating algal nuclear DNA into various sea slug chromosomes. The extent of algal gene transfer can only be determined once a complete and properly annotated *E. chlorotica* genome becomes available.

The route by which the gene transfer occurred is also unclear. There are various proposed methods in which DNA can be transferred between eukaryotic species such as direct environmental contact between donor DNA and recipient germ line cells, food ingestion when the reproductive structures are in close proximity to digestive structures or by retroviral gene movement from a donor to a host species (Boto, 2014). It is doubtful that direct environmental contact could be a route of gene transfer from *V. litorea* to *E. chlorotica*, since germ line cells are housed inside the slug and algal filaments would not have direct contact with them. In addition, direct contact between algal DNA and slug zygotes would also be quite difficult since individual zygotes are surrounded by an egg capsule and collectively, the zygotes from that clutch are enclosed by a thick jelly-coat matrix (West, 1979).

A more plausible route of transmission of algal DNA into the slug genome may be from algal food ingestion. The slug's ovotestes are directly adjacent to its digestive tubules (Rumpho *et al.*, 2009). Fragments of algal DNA derived from the mechanics of digestion could come in

contact with germ line cells if the barrier between the ovatestes and digestive tubules became compromised. In theory, this method of gene transfer would facilitate the incorporation of larger pieces of DNA into the slug's genome. Another possible manner that genes could have been transferred to the slug is via exogenous or endogenous retroviruses. Retroviruses are known to move genes between donor and host and retroviral vectors are currently used to transfer genes *in vitro* and facilitate gene therapy (Niederer and Bangham, 2014; Fablet, 2014). Natural and cultured populations of *E. chlorotica* die in the late spring which is thought to be a result of the expression of an endogenous retrovirus that leads to cell apoptosis (Pierce *et al.*, 1999; Mondy and Pierce, 2003). If the initial retrovirus originated from *V. litorea*, and then became stably integrated into the slug genome, it could have carried algal nuclear genes and transferred them to the slug. Another possibility is that *E. chlorotica*'s endogenous retrovirus may have reverse transcribed algal mRNA templates into cDNA which were then incorporated into the slug's genome. The latter may explain why intronless copies of the algal nuclear genes, *prk*, *Lhcv-3* and *chlG*, have been identified in veliger larvae gDNA (Rumpho *et al.*, 2009; Schwartz *et al.*, 2010). Although the two proposed routes of HGT from the algal nuclear genome to the slug genome are viable possibilities, they need to be experimentally proven in future studies to gain a clearer understanding of how and how many genes have been transferred.

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