Running Head: Plastid maintenance in a metazoan

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Title:  Update on Sea Slug Kleptoplasty and Plastid Maintenance in a Metazoan

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Trench (1969) was the first to characterize the kleptoplastic (i.e., “stolen plastid”) relationship between the sacoglossan mollusc *Elysia chlorotica* Gould and its algal prey (*Vaucheria litorea* C. Agardh CCMP2940). In contrast to *E. chlorotica* that retains only the plastids of the alga in densely packed digestive tissue (Fig. 1), aquatic invertebrates (e.g., corals, clams, worms, tunicates) and the recently reported spotted salamander (*Ambystoma maculatum*) (work of R. Kerney, reported by Petherick, 2010) owe their photosynthetic capacity to the retention of intact unicellular algae (reviewed in Rumpho et al., 2011). Photosynthetic sacoglossans vary in the ability to retain plastids and to maintain their functions. Whereas some of these animals can only utilize transferred photosynthate for several hours before the plastids are degraded, others sustain plastid function for months (see reviews in Rumpho et al., 2006; Händeler et al., 2009; Yamamoto et al., 2009; Rumpho et al., 2011). *E. chlorotica* exhibits one of the longest time frames for plastid maintenance in the absence of algal food; i.e., up to 10 to 12 months in the laboratory (reviewed by Rumpho et al., 2011). The obvious question to be asked about this system is how the plastids remain photosynthetically active in the absence of algal nuclei that are presumably required to furnish transcripts for plastid-targeted proteins involved in photosynthesis, signaling, regulation, and protein turnover. This update will compare and contrast past approaches used to understand the basis of plastid maintenance and function with recent work using next-generation sequencing to reconcile what appear to be contradictory outcomes in the observed data.

**Functional Sea Slug Kleptoplasty**

The exploitation of photosynthesis by heterotrophic organisms is well documented in aquatic (e.g., Trench, 1993; Venn et al., 2008) and terrestrial ecosystems (e.g., Nash, 2008). In these opportunistic relationships, the algal symbiont gains refuge and a stable nutrient source in exchange for supplying the host (e.g., invertebrate, amphibian, plant) with carbon (Trench, 1993; Yellowlees et al., 2008). Symbiotic sacoglossans develop a similar relationship, however exploiting only the plastid captured from specific algal prey (Jenson, 1980; Marin and Ros, 2004; Händeler et al., 2009). This phenomenon was recognized in *Elysia* sp. by Kawaguti and Yamasue (1965) and Trench (1969), followed by a description of the ecology (Hinde and Smith, 1974; Jenson, 1986; Clark et al., 1990) and development (Harrigan and Alkon, 1978; West, 1979; West et al., 1984). *E. chlorotica* has an obligate relationship with *Vaucheria* species,
feeding only on *V. litorea* (see Fig. 1) or *V. compacta* (West et al., 1984). Development is predominantly planktonic and the deposited eggs and planktonic larval veligers lack plastids. Growth of the veligers occurs by feeding on microalgae in the environment and metamorphosis occurs after 10 to 21 d in the water column. However, settlement and metamorphosis of the veligers into adult sea slugs requires the presence of *Vaucheria*. Most often, the veligers settle on the algal filaments ensuring a food supply for recently metamorphosed juveniles (West, 1979). Growth and maturation of *E. chlorotica* is dependent on feeding on *Vaucheria* for about one week, after which plastids are able to support continued growth of the animal (Rumpho et al., 2011). The mechanisms that allow long-term plastid photosynthetic ability in a heterotrophic host have been studied in detail but remain enigmatic.

**Hypotheses to Explain the Enigma**

**Retention of algal nuclei.** A reasonable explanation for long-term plastid maintenance is the presence of algal nuclei within the animal that provide the transcripts needed to support plastid functions. Analysis of adult, green, kleptoplastic sacoglossans using microscopy, PCR and Southern blot analysis has however failed to substantiate this hypothesis (Graves et al., 1979; Mujer et al., 1996; Green et al., 2000; Rumpho et al., 2001; Mondy and Pierce, 2003; Pierce et al., 2003; Wägele et al., 2010). In addition, molecular markers for nuclear encoded algal genes (e.g., internal transcribed spacer 1 [ITS1] and spermidine synthase [SPDS]) are not found with PCR using DNA derived from starved animal tissue (Pierce et al., 2007; Rumpho et al., 2008; Pierce et al., 2009; Schwartz et al., 2010).

**Plastid genetic autonomy.** Another potential explanation for long-term plastid function in *E. chlorotica* is the existence of a genetically autonomous *V. litorea* plastid genome. It is formally possible that this genome has regained, *via* horizontal gene transfer (HGT), critical genes encoding plastid proteins involved in photosynthesis that have been transferred to the nucleus in other algae and plants. To address this idea, Rumpho et al. (2008) sequenced the plastid genome of *V. litorea* and found it to be comparable to other algal and plant plastids in terms of gene content and found no evidence for HGT.
Plastid stability. Although containing a typical algal genome, the *Vaucheria* plastid exhibits unique physical and biochemical properties that may play a role in the establishment of the sacoglossan symbiosis (see also Jensen, 1980; Handeler et al., 2009; Wägele et al., 2010). *Vaucheria*, like many of the algae consumed by sacoglossan molluscs, is filamentous and coenocytic; in essence a single multi-nucleate cell. This allows the sea slug to rapidly acquire numerous plastids while feeding, but also favors plastids that appear to have a greater longevity than those associated with multicellular algae. Green et al. (2005) investigated the physiology of isolated *Vaucheria* plastids and found that 30 to 40% (in light vs. dark, respectively) remained intact 14 d after isolation from the alga, whereas <20% of isolated spinach chloroplasts were intact after 24 h. Isolated *V. litorea* plastids exhibited electron transport activity, CO2-dependent-O2 evolution, and CO2 fixation 72 h post-isolation. The isolated plastids were also largely unaffected by osmotic fluctuations, tested up to ±70 mM mannitol. In addition, over a 3 d period, de novo synthesis of plastid proteins in isolated plastids changed minimally in banding patterns and intensity on SDS-PAGE gels, and both Rubisco (large and small subunits) and the photosystem II D1 protein were synthesized in plastids 3 d post-isolation. These data support the unique nature of *V. litorea* plastids in either not requiring sigma factors or other nuclear encoded regulatory factors for transcription and translation of plastid genes, or that the factors are stable for a minimum of 3 d in vitro.

Dual-targeting of proteins. Dual targeting of animal-derived proteins as an explanation for plastid replenishment is yet to be carefully studied. All of the nuclear-encoded enzymes of the Calvin-Benson carbon reduction cycle have cytosolic counterparts in *E. chlorotica* except for phosphoribulokinase (PRK) and sedoheptulose-1,7-bisphosphatase (both subunits of Rubisco are plastid encoded in *V. litorea*). In addition, transcripts for all of these nuclear-encoded enzymes are present in the partial *E. chlorotica* transcriptome library discussed in more detail below (also see Table S1). Because these proteins are encoded in the animal genome, they could be co-opted for use in the plastid, providing much of the necessary machinery for carbon fixation.

Horizontal gene transfer. The most highly cited hypothesis to date, and the focus of this update, is extensive HGT from the algal nucleus to the animal, thereby supporting long-term plastid function (see also Olendzenski and Gogarten, 2009; Bock, 2010; Boto, 2010; Moran and Jarvik,
The majority of the existing work (Table I and references below) has investigated the presence of genes required for photosynthesis in the nuclear genome and transcriptome of *E. chlorotica* in both adults and larvae (with and without plastids present, respectively). Until recently, these studies used the candidate gene approach to putatively identify alga-derived genes in the animal. The methods have included PCR (Green et al., 2000; Mondy and Pierce, 2003; Pierce et al., 2003; Pierce et al., 2007; Rumpho et al., 2008; Pierce et al., 2009; Rumpho et al., 2009), detection of *de novo* synthesis of nuclear-encoded plastid proteins using radiolabeling and immunolabeling in combination with specific inhibitors of transcription or translation (Pierce et al., 1996; Green et al., 2000; Hanten and Pierce, 2001; Rumpho et al., 2001; Pierce et al., 2007; Rumpho et al., 2009), Northern blot analysis and genome walking (Rumpho et al., 2008), 14C incorporation and synthesis of chlorophyll in animal tissue (Pierce et al., 2009), and quantitative RT-PCR of nuclear encoded algal genes in the animal (Soule, 2009). All of these data support the presence of a few (Rumpho et al., 2008; Rumpho et al., 2009) to numerous (Pierce et al., 2009, Schwartz et al., 2010) nuclear algal genes or gene fragments in *E. chlorotica*.

Recently, partial transcriptome data from two kleptoplastic sea slugs, *E. timida* and *Plakobranchus ocellatus*, were generated by Wägele et al. (2010). Both of these sea slugs feed on multiple chlorophycean algae in nature and retain functional plastids for about 57 and 69 d, respectively (Evertsen et al., 2007). Analysis of 77,648 ESTs from a single *P. ocellatus* individual and 24,200 ESTs from 15 *E. timida* individuals showed that 96 to 98% of the expressed genes were metazoan, and none were of algal provenance. Based on these data, the authors concluded that HGT plays no role in sustaining long-term plastid function in these sea slugs. Rather, they attributed this unique relationship to the physical characteristics of the algal plastids and the morphology and cellular environment of the animal (physically and biochemically protecting the plastids), based on their ultrastructural, PAM fluorescence, and phylogenetic analyses (Wägele and Johnsen, 2001; Evertsen et al., 2007; Evertsen and Johnsen, 2009; Händeler et al., 2009).

Using essentially the same approach, we generated partial transcriptome data from *E. chlorotica* to investigate the extent of HGT within this system (see SI Methods, Table S1, and http://dblab.rutgers.edu/home/downloads/ to download a complete list of the isotigs and singletons produced by GS Assembler). Unlike the sacoglossans studied by Wägele et al. (2010), cumulative data (summarized above and in Table I) resulting from multiple experimental
approaches and investigators over many years have supported HGT in the *E. chlorotica/V. litorea* system, although the extent of this transfer has been in question: e.g., several key genes (Rumpho et al., 2008; Rumpho et al., 2009) or entire metabolic pathways (Pierce et al., 2009; Schwartz et al., 2010). Using 454 pyrosequencing we generated 148 Mbp of cDNA sequence data from starved, but actively photosynthesizing adult *E. chlorotica* (*n*=5). From this partial transcriptome, 13,978 assembled unigenes and 99,873 unassembled singletons were analyzed using BLASTx (*e*-value cut off ≤ 10^{-10}) to generate putative gene annotations and to assign the taxonomic origin of ESTs. As expected, at least 95% of the predicted proteins had top hits to Metazoa. The putative non-metazoan top hits returned by BLASTx analysis of the *E. chlorotica* unigenes (123) and singletons (354) were used as queries in a phylogenomic pipeline specifically designed to identify genes of foreign origin from the host using the methods and database described in Moustafa et al. (2009). This approach identified 20 ESTs of potential foreign origin derived from different prokaryotes, eukaryotes, and viruses (Table S2), as well as several plastid-derived transcripts primarily from *V. litorea*, indicating plastid activity (Table S3). None of these 20 ESTs, however, have a direct involvement in photosynthesis. In addition, specific BLASTn searches of both the contig and singleton data for genes previously identified as HGT candidates (*lhcv* 1,2,3,4; *fcp*; *psbO*; *prk*; *uroD*; *chlD,H,G*; see Table I), failed to identify homologs.

In all of these transcriptome studies, no evidence exists to indicate that nuclear-encoded algal genes are expressed in photosynthetic sacoglossans. Wägele et al. (2010) noted the high expression level of photosynthetic genes in the transcriptome of the source alga *Acetabularia*, comparable to that observed in the model green plant *Arabidopsis*, and yet none of the “top 50” highly expressed nuclear transcripts for plastid protein were found in the two sacoglossans used in their study. Although data are not publicly available from the *V. litorea* transcriptome to conduct such a comparative analysis (Pierce et al., 2009), one can assume that a similar hierarchy of expression patterns would be observed in this alga. In the partial transcriptome data presented here from *E. chlorotica*, we also did not find evidence of expression of any of the photosynthetic “top 50” nuclear encoded algal genes. However, it is important to note that expression patterns of a putatively transferred gene in the animal may not mirror what is observed in the alga, and to assume similar expression patterns in the foreign environment is highly speculative. Not only might copy number of the genes be markedly different but the expression levels and modes of regulation of expression in the animal are unknown. Recent studies employing quantitative RT-
PCR support expression of both prk and psbO in starved E. chlorotica. However, the expression levels of these genes in the animal were markedly lower, as much as 525-times lower for prk (Soule, 2009) and 63-times lower for psbO (unpublished data, Soule and Rumpho) compared to expression levels measured in V. litorea under identical (2 h-post illumination) conditions. Differential expression of both prk and psbO in the animal was observed over a diurnal cycle, but again these patterns were markedly different from that observed in V. litorea. Similar patterns were also observed with the plastid-encoded transcripts rbcL and psaA. These studies provide evidence of expression of nuclear-encoded plastid targeted proteins in E. chlorotica starved for several months, but more importantly, they suggest that levels and patterns of expression of foreign genes may not mirror those in the alga (e.g., Acetabularia or Vaucheria). Thus, although Wägele et al. (2010) provide compelling arguments to suggest that nuclear-encoded algal transcripts could not have been missed in their transcriptome libraries, we feel more exhaustive sequencing may be required to adequately address this issue.

Of interest in both studies is the finding of plastid gene expression, indicating this genome is actively transcribed in the animals. The 46 plastid ESTs identified included 19 unique genes in E. chlorotica, some of which were represented multiple times (Table S3). Together with the data from Wägele et al. (2010), these data suggest that nuclear encoded transcription factors or regulatory molecules are not necessarily required for plastid gene expression in these taxa.

**Future Directions**

Alternate hypotheses to HGT need to be considered to explain plastid function in sacoglossans. It is apparent that plastid stability is essential for the success of these symbioses, from initial uptake to establishment of kleptoplasty (Green et al., 2005; Wägele et al., 2010). The extent to which HGT is occurring is less clear and warrants further exploration to resolve the conflicting results to date. Wägele et al. (2010) conclude that HGT does not explain plastid longevity and function in the two sacoglossans investigated in their study. A similar conclusion cannot yet be drawn for E. chlorotica due to the longevity of this functional association and multiple lines of evidence that indicate nuclear algal genes have been transferred. Therefore, either these sacoglossan species (E. chlorotica vs. E. timida and P. ocellatus) have evolved unique mechanisms to obtain and sustain photosynthetic abilities, one utilizing gene transfer and the others not, or additional explanations are required to reconcile current data.
Finally, reconciliation of these data will only occur through the investigation of new mechanisms that could be working synergistically with limited HGT and plastid stability towards plastid function. This could include investigating whether: 1) the animal cells transiently use proteins and transcripts in an opportunistic fashion when available, 2) transcripts are integrated into extra-chromosomal elements which may restrict detection due to extremely low copy number and expression levels, 3) the unique stability of the plastids translates to unusual protein turnover rates of essential plastid- and nuclear-encoded photosynthetic proteins in the animal vs. alga and/or protease activity, and 4) the regulation of expression of foreign genes in the animal impacts traditional detection approaches.

**Supplemental Material**

SI Methods.

Table S1. List of metazoan-derived unigenes and their top hits found using BLASTx and our comprehensive local database. The gene ontology (GO) annotations are also presented.

Table S2: ESTs of putative non-metazoan origin identified in unigenes and singletons derived from an *Elysia chlorotica* partial transcriptome.

Table S3: ESTs of plastid origin from the singletons of the *Elysia chlorotica* transcriptome library.
Literature Cited


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West HH (1979) Chloroplast symbiosis and development of the ascoglossan opisthobranch Elysia chlorotica. PhD thesis. Northeastern University, Boston


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1 Where identical data were presented in more than one publication, the earliest publication date was noted.
2 ND = Not determined.
Figure 1. A) Adult *Elysia chlorotica* feeding on its algal prey, the coenocytic heterokont *Vaucheria litorea* (bar = 5 mm). B) Confocal micrograph showing the red autofluorescent plastids densely packed within the digestive diverticula of *E. chlorotica* (bar = 50µm). Once established, these plastids remain photosynthetically active within the adult for several months.