The Journal of Experimental Biology 214, 303-311

doi:10.1242/jeb.046540

The making of a photosynthetic animal

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Accepted 6 August 2010

Summary

Symbiotic animals containing green photobionts challenge the common perception that only plants are capable of capturing the sun's rays and converting them into biological energy through photoautotrophic CO_2 fixation (photosynthesis). 'Solar-powered' sacoglossan molluscs, or sea slugs, have taken this type of symbiotic association one step further by solely harboring the photosynthetic organelle, the plastid (=chloroplast). One such sea slug, *Elysia chlorotica*, lives as a 'plant' when provided with only light and air as a result of acquiring plastids during feeding on its algal prey *Vaucheria litorea*. The captured plastids (kleptoplasts) are retained intracellularly in cells lining the digestive diverticula of the sea slug, a phenomenon sometimes referred to as kleptoplasty. Photosynthesis by the plastids provides *E. chlorotica* with energy and fixed carbon for its entire lifespan of ~10 months. The plastids are not transmitted vertically (i.e. are absent in eggs) and do not undergo division in the sea slug. However, *de novo* protein synthesis continues, including plastid- and nuclear-encoded plastid-targeted proteins, despite the apparent absence of algal nuclei. Here we discuss current data and provide hypotheses to explain how long-term photosynthetic activity is maintained by the kleptoplasts. This fascinating 'green animal' provides a unique model to study the evolution of photosynthesis in a multicellular heterotrophic organism.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/2/303/DC1

Key words: *Elysia chlorotica*, horizontal gene transfer, mollusc, photosynthesis, plastid, sea slug, symbiosis, *Vaucheria litorea*.

Introduction

It has long been known that photosynthesis takes place in chlorophyll-containing plants, algae and some bacteria and that it serves to provide oxygen and energy in the form of biomass to support heterotrophic life. Some animals (for the purposes of this review we will address only the metazoans) in the phyla Mollusca (giant clams, nudibranchs), Porifera (sponges), Cnidaria (corals, anemones and hydra), Acoelomorpha (flatworms) and Chordata (ascidians) have evolved mechanisms to capture photosynthetic products through the formation of symbiotic associations with intact unicellular algae or cyanobacteria (Fig. 1 and supplementary material Table S1 and references therein). In these cases, the photobiont (alga or cyanobacterium) acts as an autonomous photosynthetic factory, providing reduced carbon as a source of energy to the heterotroph, often receiving nutrients in return. Whereas initial events leading to primary photosynthetic eukaryotes involved phagocytosis by a unicellular protist (see below), the acquisition of photosynthetic symbionts by multicellular animals poses multiple challenges. Among these are: localization of the symbiont to differentiated tissues, regulation of the symbiont environment, protein/metabolite transfer and turnover, evasion or suppression of the host immune response and, in order for the symbiosis to become permanent, transmission of the symbiont to the germline (Venn et al., 2008; Raven et al., 2009).

The ability of animals to acquire photobionts appears limited to aquatic environments and to the few phyla mentioned above. The morphology of these multicellular organisms is an important factor leading to such relationships. Photosynthetic animals tend to exhibit simple morphologies (in many, only two cell layers) coupled with a large surface area to volume ratio to accommodate the organelles and transfer of photosynthates and other nutrients (Venn et al., 2008) (see examples in Fig. 1A–C). Other apparently adaptive modifications are seen in the morphologically advanced molluscs (bivalves, nudibranchs and sacoglossans), in which the finely branching diverticula of the digestive tract achieves a similar high surface area to volume ratio in the more complex tissue of these hosts (see examples in Fig. 1G and Fig. 2) (Graves et al., 1979; Norton et al., 1992; Venn et al., 2008). Within the host animal, symbiotic algae and cyanobacteria are either compartmentalized within a host membrane (e.g. symbiosomes in cnidarians) or localized to a specific region (e.g. the heamal sinus of tridacnid clams or the epidermal surface of certain ascidians). The symbionts are also typically restricted to regions of the body cavity that enable the most efficient light capture. Often, morphological adaptations by the host facilitate symbiont light capture, compensating for the loss of mobility characteristic of the algal symbionts (Trench, 1993). The membrane-bound compartment poses a greater challenge to the transfer of materials between host and symbiont, but conversely provides a more controlled environment. Numerous studies have addressed the dynamics of metabolite and protein exchange between host and symbiont; in many cases both partners have evolved adaptations to support the relationship (reviewed by Trench, 1993; Yellowlees et al., 2008; Venn et al., 2008). Whether symbionts overcome or avoid innate immune responses of the host (all photosynthetic animals studied to date have only innate immunity) is still largely unknown, and the mechanisms are not

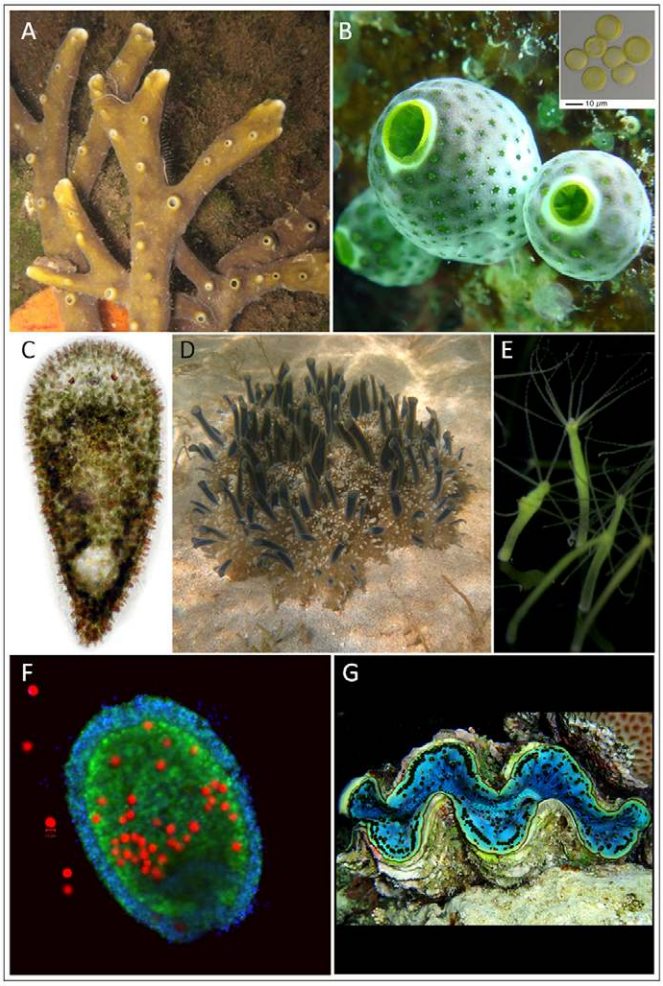

Fig. 1. Examples of diverse photosynthetic animals with varied symbionts. (A) *Neopetrosia subtriangularis* with *Synechococcus* (photo by Robert Thacker); (B) *Didemnum molle* with *Prochloron* (inset) (photo by Euichi Hirose); (C) *Symsagittifera* sp. with *Tetraselmis* symbionts [modified here from fig. 1 in Hooge and Tyler (Hooge and Tyler, 2008), with permission of Magnolia Press]; (D) *Cassiopea xamachana* with *Symbiodinium* (photo by Alan Verde); (E) green *Hydra* with *Chlorella* (photo by Thomas Bosch); (F) confocal image of *Fungia* coral larva (blue) with red autofluorescent *Symbiodinium* (photo by Virginia Weis); and (G) *Tridacna* spp. with *Symbiodinium* (photo by Jesús Pineda with permission from Woods Hole Oceanographic Institution).

understood. However, recent transcriptomic work studying coral–algal symbioses has shown that gene expression of the host, when infected by the appropriate symbiont, shows little or no change over time (Voolstra et al., 2009). By contrast, when the host is exposed to foreign symbionts (those incapable of establishing a symbiosis) significant changes in the host transcriptome result and the immune response is launched. This difference implies that appropriate symbionts evade detection by the host and fail to elicit recognition, rejection and immune responses such as apoptosis and proteolysis (Voolstra et al., 2009). Further studies of the immune response in other photosynthetic animals are needed to determine whether similar mechanisms are employed across all of these organisms. Transmission of photosynthetic symbionts is largely horizontal, with each generation acquiring its photosynthetic partner anew from the surrounding environment. Transmission to the germline remains a final barrier towards a more permanent photosynthetic animal.

In an even more unusual adaptation leading to photosynthesis by animals, several sacoglossan molluscs (sea slugs), particularly in the genus *Elysia*, have evolved the ability to retain only the functional plastids from their algal prey (Fig. 2 and supplementary material Table S2 and references therein). The plastids are retained intracellularly in cells lining the animal's digestive diverticula and remain photosynthetically active for varying lengths of time depending upon the host–symbiont association (reviewed in Rumpho et al., 2000). Reports on the presence of green pigment and then green 'animals' in sacoglossans were made in the late 1800s (De Negri and De Negri, 1876; Brandt, 1883) [as reported in Clark et al. (Clark et al., 1990)]. The discovery that the green 'bodies' were plastids was made in 1965 after microscopic observations of *Elysia atroviridis* (Kawaguti and Yamasu, 1965). Research publications over the next 10–20 years, principally by Trench (Trench, 1969; Trench et al., 1969; Trench et al., 1972; Trench et al., 1973a; Trench et al., 1973b; Muscatine et al., 1975; Trench, 1975), but also by Clark (Clark and Busacca, 1978; Clark et al., 1990), Jensen (Jensen, 1986), Hinde (Hinde, 1980; Hinde and Smith, 1972; Hinde and Smith, 1974) and others (Green and Muscatine, 1972; Graves et al., 1979), greatly advanced our understanding of functional kleptoplasty in sea slugs, in particular at the ecological and physiological levels.

Many studies have employed a variety of methods to characterize the duration of photosynthesis in these sacoglossans, often generating conflicting results for many species (supplementary material Table S2). These contradictions warrant more thorough investigations of photosynthesis *in situ*. Phylogenetic studies have been more helpful in elucidating the evolution and distribution of the photosynthetic abilities of these animals. Händeler et al. investigated the phylogeny of numerous sacoglossans coupled with functional photosynthesis (Händeler et al., 2009). Their work supports the evolution of short-term plastid retention in sacoglossans once in a common ancestor of the Plakobranchoidea, and the lack of plastid retention in members of the other superfamilies (Oxynoacea and Limapontioidea). Subsequently, at least four species of sacoglossans independently evolved the ability for long-term plastid retention. Morphology of these sacoglossans is thought to play a role in long-term plastid retention; animals with wing-like parapodia are able to regulate light exposure and have evolved the ability to maintain these plastids for several months (Händeler et al., 2009) (Fig. 2A–C). In *E. chlorotica*, one of the four derived sacoglossans with parapodia (Fig. 2A), the captured plastids can serve as the sole energy source for the animal for over 10 months (Green et al., 2000; Rumpho et al., 2006). These photosynthetic animals are closer to a permanent symbiosis in that the organelle is intracellular and is often not bound by a host-derived membrane. Control of the plastid processes remains enigmatic (discussed below).

Evolution of plastids and photosynthetic organisms

Photosynthetic eukaryotes reflect an evolutionary history of close physical contact between organisms leading to multiple endosymbiotic events, gene transfer and the evolution of modern-day mitochondria and plastids. Mitochondria owe their origin to the uptake of an α-proteobacterium (Gray et al., 2001), and plastids to the subsequent uptake of a photosynthetic cyanobacterium (Reyes-Prieto et al., 2007). These two key primary endosymbioses led to free-living prokaryotes becoming highly dependent upon their host nucleo-cytosol because genes

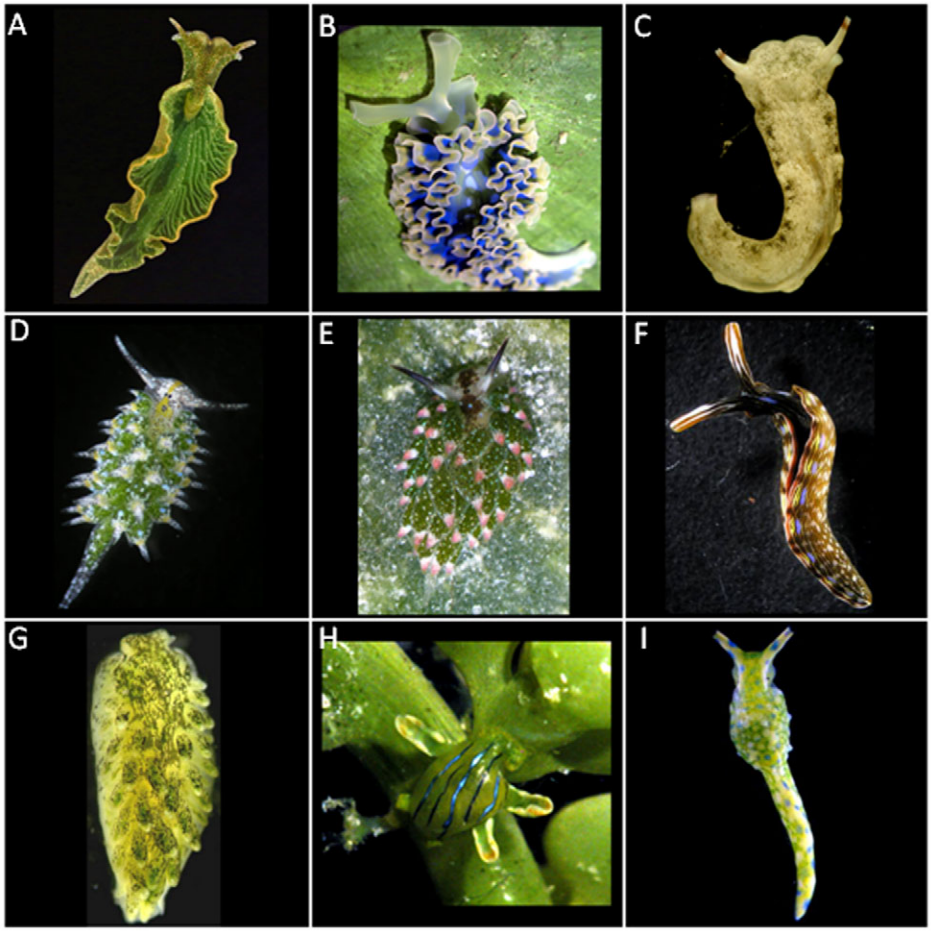

Fig. 2. Examples of photosynthetic sacoglossans with varied times of chloroplast retention. (A) *Elysia chlorotica* [reprinted with permission (Rumpho et al., 2008)], (B) *Elysia crispata*, (C) *Plakobranchus ocellatus*, (D) *Costasiella ocellifera*, (E) *Thuridilla gracilis*, (F) *Costasiella kurishimae*, (G) *Alderia modesta*, (H) *Lobiger viridis* and (I) *Oxynoe antillarum*. Photos in panels B, D, E, G and I were provided with permission by Patrick Krug; photos in panels C, F and H were provided with permission by Heike Wägele.

were lost or transferred to the nucleus in the first examples of massive intracellular gene transfer. The ancestral photosynthetic organism gave rise to three primary lineages, the glaucophytes, the rhodophytes (red algae) and the Chloroplastida or Viridiplantae (green algae and land plants) (Bhattacharya and Medlin, 1995; Falkowski et al., 2004; Adl et al., 2005) (see evolutionary scheme in Fig. 3). In turn, a diverse group of secondary or 'complex' algae evolved following the engulfment of a single-celled green or red alga (or both) by a heterotrophic, eukaryotic host (McFadden, 2001). Genome modification again occurred as a result of gene transfer and loss from the symbiont plastid, but also as a result of the 'merger' of the two nuclei (host and endosymbiont) (reviewed in Lane and Archibald, 2008). The evolution of photosynthetic organisms did not stop here, however. Serial secondary endosymbiosis, the replacement of the original primary plastid by a different primary plastid, and tertiary endosymbiosis, the engulfment of a secondarily derived plastid by a heterotrophic or autotrophic eukaryotic host (Bhattacharya and Nosenko, 2008), gave rise to additional algal diversification and genome chimerism (Keeling, 2004; Yoon et al., 2005; Lane and Archibald, 2008; Sanchez-Puerta and Delwiche, 2008). In organisms that evolved through serial endosymbiosis, previously transferred plastid genes would have been present in the host nuclear genome, but could have also been replaced by plastid or nuclear genes of the new symbiont. In the case of tertiary endosymbiosis, genes required to support functions of the most recently acquired plastid may have been present in the host from a previous endosymbiotic partner, or they may also have been transferred from the newest endosymbiont's nuclear and plastid genomes to the host's nuclear genome (Lane and Archibald, 2008). Finally, recent data suggest that stramenopiles and other chromalveolates share a cryptic green algal endosymbiont that predates the canonical red algal capture that has also contributed significantly (100s of genes) to their nuclear genome (Moustafa et al., 2009). Therefore, the genomes of many chromalveolate taxa (minimally) contain genes of red and green algal origin derived from eukaryotic endosymbioses (Moustafa et al., 2009).

Evolution of kleptoplasty and photosynthesis in *Elysia*

To the extent that it has been studied, the sea slug *Elysia chlorotica* Gould 1870 is specific for its algal prey, feeding on and acquiring plastids with any success from only two *Vaucheria* species (*V. litorea* and *V. compacta*) (West, 1979; West et al., 1984). Not only is the association specific, but it is also obligate; the sea slug will not complete metamorphosis and develop into an adult in the absence of its algal prey and plastid uptake. *Vaucheria* is a member of the stramenopiles that currently contain red algal secondary-derived plastids. Biochemically, the alga is characterized by chlorophylls *a* and *c*, vaucheriaxanthin accessory pigments, lipid carbon reserves (Anderson, 2004; Lee, 2008) and high mannitol levels (M.E.R., unpublished data). Its coenocytic (single-celled, multi-nucleate) filaments are largely vacuolated and surrounded by a thin cell wall. As a result of secondary plastid evolution, these organelles are surrounded by four membranes in *V. litorea* (Rumpho et al., 2001): the inner and outer envelopes, the periplastid membrane (a remnant of the plasma membrane of the engulfed alga) and the outermost plastid endoplasmic reticulum membrane (Gibbs, 1993; Bhattacharya et al., 2004). Interestingly, the outer

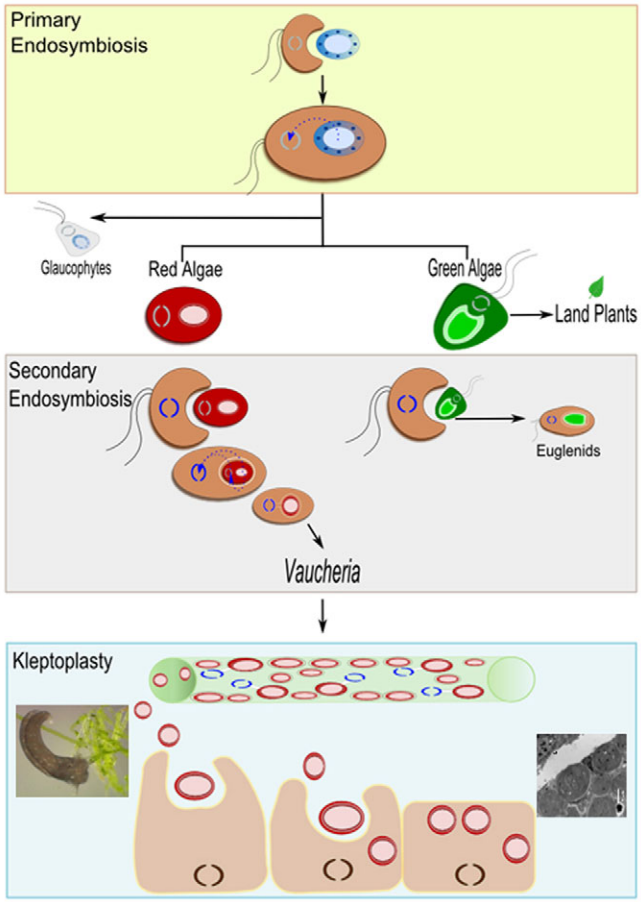

Fig. 3. Evolutionary scheme for primary, secondary and tertiary plastids. The secondary endosymbiotic origin of plastids is illustrated in *Vaucheria litorea* from the red algal lineage. The subsequent acquisition of *V. litorea* plastids by the sea slug *Elysia chlorotica* in a tertiary endosymbiotic event imparts photosynthetic activity to this heterotroph.

two membranes are not readily observed in the sea slug (Rumpho et al., 2001) and this has potential implications for protein targeting, but will not be discussed here.

Because *E. chlorotica* obtains its plastids from a eukaryote containing plastids of secondary origin, the evolution of plastid retention (kleptoplasty) and photosynthesis in the sea slug can be explained in a parallel fashion to that of other tertiary-evolved photosynthetic organisms encompassing both endosymbiosis and potentially horizontal gene transfer (HGT; defined as the non-sexual exchange of genetic material) as follows. (1) The heterotrophic eukaryotic host and symbiont establish close, prolonged physical contact; i.e. the host sea slug (*E. chlorotica*) grazes on mats of the algal prey (*V. litorea*), sucking out the cellular contents (including intact and broken plastids, mitochondria and nuclei), which then slowly traverse the digestive gut. (2) The symbiont is taken up by the host; i.e. algal plastids in the digestive gut are phagocytosed by the digestive epithelial cells of the host sea slug. (3) Genome modification occurs; i.e. gene transfer may have occurred through HGT from the kleptoplasts in the epithelial cells and from broken algal nuclei releasing DNA in the gut lumen to the host nucleus. (4) New metabolic properties evolve as a result of the kleptoplastic association; i.e. an animal (*E. chlorotica*) is able to sustain itself solely by photoautotrophic CO_2 fixation, as a plant.

There are two major differences to consider in this comparison. First, the entire *V. litorea* algal cell, including intact nuclei, is not intracellularly taken up into the host sea slug. Second, the kleptoplastic association is not transmitted vertically. In the first case, the absence of the algal nucleus in the sea slug cell has significant implications for providing nuclear-encoded plastid proteins on a long-term basis. In the second case, absence of heritability is most likely due to a soma–germline barrier that could prevent the movement of plastids into the invertebrate germline and, hence, into the progeny. Whether the sea slug is 'on the path' to the evolution of permanent photosynthesis is unknown, in large part because of the lack of genome data.

Laboratory culturing of kleptoplastic sea slugs

To date, most of the studies on *E. chlorotica* have been carried out on adult kleptoplastic animals collected from the ocean. The 'true' age of the sea slugs, the extent of feeding, the history of the population and sea slug behavior (e.g. mating) are unknown in these collections. Furthermore, because the association is already established we cannot explore the early steps in development, uptake and retention of the plastids or the initiation of photosynthesis. Virtually nothing is known at the biochemical or molecular level about any of these processes. Finally, we also do not know what contributes to the great disparity in longevity of the different kleptoplastic associations or, in particular, their photosynthetic activity.

The successful establishment of a laboratory culture system has provided us the necessary controls to fully investigate sea slug development and establishment of kleptoplasty and photosynthesis. We optimized an artificial saltwater (ASW) culture system using aposymbiotic eggs produced by *E. chlorotica* populations from Martha's Vineyard Island, MA, USA, and near Halifax, Nova Scotia (see life cycle in Fig. 4). Successful planktotrophic development was recorded for all developing larvae that were fed a unicellular algal diet of *Isochrysis galbana*. Metamorphosis of larvae to the juvenile stage requires the presence of *V. litorea* filaments. Immediately following metamorphosis, the juveniles begin feeding on the filamentous alga, engulfing plastids and turning green. A transient nature to the plastid symbiotic association is observed in recently metamorphosed juvenile sea slugs if removed from the presence of *V. litorea* too soon (less than ~6 days); this also results in cessation of their morphological development. Plastid uptake until the establishment of irreversible kleptoplasty appears to be required for full adult development and survival, although one report of 'albino ghost' *E. chlorotica* was documented in 1986 (Gibson et al., 1986). Establishment of the kleptoplastic association involves specific recognition processes that comprise at least two steps: (1) planktonic larvae require *V. litorea* filaments to be present for settlement and metamorphosis to the juvenile stage, and (2) adult development requires uptake and retention of *V. litorea* plastids by cells lining the digestive diverticula.

Semi-autonomy of plastids and the need for the nucleus

Endosymbiosis and the associated gene transfer that followed rendered extant plastid genomes greatly reduced in size (37–224 kb), encoding between 61 and 273 proteins (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=2759&opt=plastid) compared with the cyanobacterial progenitors [1.6–9.0 Mb in free-living taxa, encoding 1717 to 7672 proteins (Meeks et al., 2001; Rocap et al., 2003)]. These semi-autonomous organelles encode a small percentage of the predicted 1000 to 5000

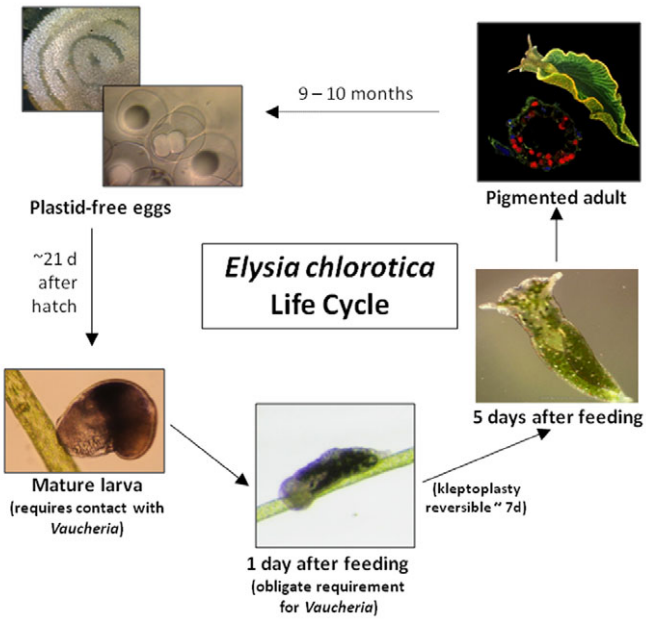

Fig. 4. Life cycle of *Elysia chlorotica*. After 4 days, veliger larvae hatch from egg ribbons and live planktonically for 3 weeks until competent for metamorphosis. Upon detection of the algal prey *Vaucheria litorea*, mature veligers settle out of the water onto the algal filaments and metamorphose into juvenile sea slugs. Feeding occurs immediately and plastids are observed inside the animal within 24 h of settlement and metamorphosis. After continual feeding of 5 to 7 days, the association becomes permanent and the plastids are stable within the animal. Additional feeding leads to growth of the juvenile to the adult stage and further incorporation of plastids into the animal tissues. Adults live for ~10 months in the wild, senescing often after mating in the spring.

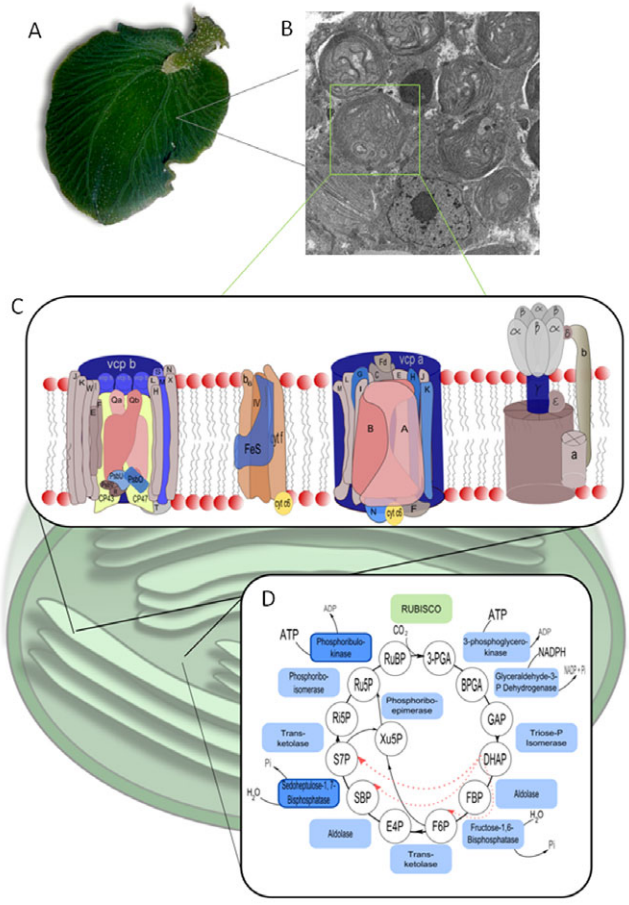

Fig. 5. Schematic of the light and dark reactions of photosynthesis showing plastid- *vs* nuclear-encoded genes. (A) Adult, kleptoplastic *Elysia chlorotica*. (B) Transmission electron micrograph showing numerous algal plastids within a cell lining the digestive diverticuli of the sea slug. (C,D) Schematic of the two photosynthetic processes overlaid on a plastid illustrating the essential proteins required in each pathway. Nuclear-encoded plastid proteins are shaded blue for both the electron transfer chain (C) and the Calvin–Bensen cycle (D). In the latter, RuBisCO is shaded green to indicate a plastid-encoded protein. Two of the enzymes, phosphoribulokinase and sedoheptulose-1,7-bisphosphatase, are shaded dark blue to indicate that, although they are nuclear-encoded like the light-blue-shaded enzymes, these enzymes are unique to phototrophs and are not typically found in an animal, whereas the light-blue-shaded enzymes all have homologs in animal metabolism.

proteins required to sustain the full metabolic capacity of the plastid (Martin et al., 2002; Richly and Leister, 2004; Bock and Timmis, 2008). This is also true for the 115-kb *V. litorea* plastid genome, which we recently sequenced, demonstrating that it contains only 139 protein-encoding genes (Rumpho et al., 2008). If we consider solely photosynthesis, the *V. litorea* plastid genome does not encode all of the components for any of the four multi-subunit complexes of the photosynthetic electron transport chain [photosystems I and II (PSI and PSII, respectively), the cytochrome b_6/f complex and ATP synthase] or the reductive pentose phosphate pathway (RPPP, or the Calvin–Benson cycle) (reviewed in Raghavendra, 1998; Nelson and Yocum, 2006). Some of the essential missing genes in the thylakoid-localized electron transport chain include: the PSI and PSII light-harvesting complex pigment/protein genes (*vcp* in *V. litorea*), the PSII Mn-stabilizing protein of the oxygen evolution complex (MSP, encoded by *psbO*), the Reiske Fe-S protein of the cytochrome b_6/f complex and *atpC*, which encodes the critical redox-regulated γ subunit of ATP synthase (see Fig. 5 schematic).

Only one enzyme of the RPPP is plastid encoded, the essential carboxylating enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Fig. 5). Unlike plants and green algae, both the large (*rbc*L) and small (*rbc*S) subunits are plastid-encoded in *V. litorea* (Rumpho et al., 2008). The other ten enzymes of the cycle are nuclear-encoded, and all but phosphoribulokinase (*prk*) and sedoheptulose-1,7-bisphosphatase (*sbp*) are also encoded by the nuclear genome of animals for glycolysis and/or the oxidative pentose phosphate pathway [see Rumpho et al. (Rumpho et al., 2008) for a complete listing of plastid-encoded genes in *V. litorea*]. As a result, it is possible that the animal could provide substitute proteins for the majority of the nuclear-encoded RPPP enzymes if they were properly targeted to the foreign plastids. The remaining two RPPP enzymes, PRK and SBP, as well as the nuclear-encoded electron transport proteins discussed above, are excellent targets for the study of HGT.

Plastids are highly evolved to absorb the intense energy of sunlight and fuel photosynthetic carbon reduction and, as a result, plastid proteins are subject to constant photo-oxidative damage by reactive oxygen species (Aro et al., 1993; Aro et al., 2005). To maintain homeostasis and uninterrupted function of the plastid, these damaged proteins must be removed [presumably by proteases (Adam and Clarke, 2002)] and/or repaired (frequently involving

chaperones) (Sakamoto, 2006); the entire process requires extensive nucleo-cytosolic communication as well as *de novo* synthesis of plastid- and nuclear-encoded plastid proteins (Biehl et al., 2005). The consequences of severing the plastid–algal nucleo-cytosolic communication network in the sea slug cells are unknown, but one would expect it minimally to result in uncoordinated plastid activity and, more likely, to the rapid demise of the organelles. What is so remarkable in this case is that the original acquired plastids sustain the starved animals for their entire lifespan (at least 10 months in nature or in the laboratory) through photoautotrophy, despite the absence of any detectable algal nucleo-cytosol in the sea slug (reviewed in Rumpho et al., 2006); this is both impressive and intriguing. We have been working under the hypothesis that the sea slug provides at least some of the essential nuclear-encoded plastid-proteins as a result of HGT from the algal nucleus to the sea slug as discussed above. More specifically, we propose that as the algal cell contents pass through the sea slug gut, DNA (and possibly RNA) released from broken nuclei may have been taken up directly or transferred by a viral vector. The foreign DNA then become part of the animal nuclear DNA, transferring genetic information from the algal nucleus to the sea slug and helping to impart new traits to the animal over the course of evolutionary time. It is also likely that transfer of plastid DNA has occurred or is still occurring as a result of rupture of some of the plastids in the sea slug cells.

Horizontal gene transfer

With the increased access to affordable high-throughput sequencing technology, evidence supporting HGT has greatly increased, including in eukaryotes (see reviews by Bock, 2010; Keeling, 2009). Along with this has emerged a greater acceptance of HGT as a mechanism for the more rapid evolution of metabolic novelty in prokaryotes and eukaryotes (Bock, 2010; Keeling, 2009; Klasson et al., 2009; Marchetti et al., 2009; Moran and Jarvik, 2010). A common theme among examples of HGT is a close physical association between host and symbiont, whether this is a result of parasitism, mutualism, phagotrophy or some other phenomenon that brings the genetic materials of the organisms in close proximity. HGT was first reported in prokaryotes, where it is abundant and of particular interest owing to the implications for human health as a result of increased bacterial resistance and virulence (Akiba et al., 1960; de la Cruz and Davies, 2000; Koonin et al., 2001; Friesen et al., 2006) and the relatively ‘easy’ acquisition of novel metabolic properties (reviewed by Sanders, 2006). Viruses can be a source as well as a vector for HGT. This was shown early on for cyanophage photosynthesis genes (Mann et al., 2003) and phosphate metabolism genes (Sullivan et al., 2005; Heinemann and Kurenbach, 2009). This was demonstrated even more broadly, covering many more physiological functions and metabolic pathways and organisms, by the microbial metagenomic analyses of the Global Ocean Sampling Expedition led by J. Craig Venter (Williamson et al., 2008).

Many of the examples of HGT between prokaryotes and unicellular (Andersson, 2005; Loftus et al., 2005; Nosenko and Bhattacharya, 2007) or multicellular eukaryotes (Nikoh et al., 2008; Starcevic et al., 2008) are associated with parasitism or phagotrophy (reviewed by Bock, 2010). The classic example of HGT from a prokaryote to a multicellular eukaryote is the transfer of DNA *via* the *Agrobacterium* Ti plasmid to plants (reviewed by Gelvin, 2009). However, more recently, HGT has been elegantly demonstrated between the *Wolbachia* bacterium and a number of invertebrates (beetles, fruit flies, mosquitoes and nematodes), and biological functionality has been shown as well in some cases (Kondo et al., 2002; Nikoh et al., 2008; Wasmuth et al., 2008; Klasson et al., 2009; Woolfit et al., 2009). A notable example of HGT, discovered through a recent whole-genomic sequencing effort, is the discovery of >500 bacterial genes in the diatom *Phaeodactylum tricornutum* (Bowler et al., 2008) and >250 in *Thalassiosira pseudonana* (Armbrust et al., 2004). In addition, the acquisition of a prokaryote ferritin gene by some pinnate diatoms appears to have given them an advantage in sequestering iron from the ocean (Marchetti et al., 2009). Thus, the incidence of HGT from prokaryotes to eukaryotes may be more prolific than once thought.

Evidence supporting the exchange of genetic material between eukaryotes is still quite rare, but new examples are being identified with high-throughput genomic sequencing (reviewed by Bock, 2010; Keeling, 2009). Most recently, Moran and Jarvik identified fungal genes in the genome of the pea aphid (Moran and Jarvik, 2010); these foreign eukaryote genes were overlooked at first because the focus was only on prokaryote gene transfer. HGT of the fungal genes yielded a new animal function, the biosynthesis of carotenoids, believed to be utilized by the aphids in mimicry to avoid predators. In another genomics study, Richards et al. identified five fungal genes in the genomes of four land plants, a bryophyte and a lycophyte (Richards et al., 2009). In turn, four plant genes were found to have been transferred to fungi. Gene transfer between two different fungi has resulted in a new, highly virulent wheat pathogen (Friesen et al., 2006). Large-scale transfer between a number of algal species and the phagocytic chlorarachniophyte alga *Bigelowiella natans* has also been detected (Archibald et al., 2003).

Exchange of mitochondrial DNA is fairly rampant between host and parasitic or epiphytic plants (reviewed in Richardson and Palmer, 2007), and entire mitochondria (Carlsson et al., 2007) and DNA have been shown to move locally through a graft junction (Stegemann and Bock, 2009). Mitochondrion to mitochondrion gene transfer is now recognized as a dominant mode of HGT in plants because of the larger and more plastic mitochondrial genomes in these taxa (Richardson and Palmer, 2007). The smaller, compact animal or metazoan mitochondrion genome is generally believed to be a poorer target for foreign gene insertion. However, some basal metazoans do exhibit greater variation in mitochondrial genome size and gene content (Lavrov, 2007). This includes multiple examples of HGT of group I intron sequences (normally not found in animals) into the mitochondrial genome of a sponge (Rot et al., 2006), a sea anemone (Beagley et al., 1996) and a coral (van Oppen et al., 2002). Non-mitochondrial HGT involving metazoans is best characterized in the bdelloid rotifer *Adineta vaga* (e.g. Gladyshev and Meselson, 2008). Two things stand out in this case: the diversity of sources of transferred genes, which included bacteria, fungi and plants, and the common insertion of the foreign genes near the telomere ends of the bdelloid chromosomes.

HGT between *V. litorea* and *E. chlorotica* was first alluded to following biochemical studies; protein radiolabeling and immunoprecipitation demonstrated *de novo* synthesis of an algal nuclear-encoded plastid light harvesting protein (VCP) in *E. chlorotica* in the presence of a plastid translation inhibitor (Pierce et al., 1996). A second line of evidence came from experiments demonstrating *de novo* synthesis of the PRK protein in sea slugs starved for several months of their algal prey (Rumpho et al., 2009), and quantitative real-time PCR measurements of changes in *prk* transcript levels in response to light induction in starved sea slugs (Soule, 2009). Amplification of algal nuclear genes encoding

plastid proteins in *Elysia* provided a third line of evidence. The focus again was on the family of light harvesting genes (*vcp*) (Pierce et al., 2007), *psb*O (Rumpho et al., 2008), *prk* (Rumpho et al., 2009) and, more recently, chlorophyll biosynthesis genes (Pierce et al., 2009; Schwartz et al., 2010). In these cases, the genes were PCR-amplified from *E. chlorotica* adult DNA and usually aposymbiotic sea slug egg or veliger DNA. Not surprisingly, none of these genes was found when we sequenced and analyzed the mitochondrial DNA (mtDNA) of *E. chlorotica* (Rumpho et al., 2008). It is more likely that large-scale gene insertion would be more readily accommodated in sea slug nuclear DNA than in mtDNA. The conclusion that the source of these genes was *V. litorea* was based on the fact that the DNA sequences from the predator and prey genomes were identical to near identical. It is logical to look for HGT targeted at *V. litorea* genes based on the physical biological association of the host and prey. However, we do not believe that *V. litorea* is the only source of HGT in *E. chlorotica*.

Claims of HGT, in particular between members of unrelated taxa, require considerable caution. One only has to look at the problems with the early reports of massive HGT in the draft human genome (Lander et al., 2001) and the later dismissal of these claims with additional sequence data and analysis (Roelofs et al., 2001; Salzburg et al., 2001). Contamination is always a concern with HGT (Willerslev et al., 2002), in particular when using PCR to detect targeted genes. As discussed above, PCR has been the primary experimental approach used in the *Elysia* system to date. Potential contamination from animal contents, including algal remnants and nuclei, is a concern. All of the experiments in the authors' laboratory were carried out on sea slugs that had not been in contact with any algal prey for several months. PCR reactions were also carried out on aposymbiotic sea slug egg DNA to exclude the possibility of algal nuclei remaining in the gut of the sea slug and contaminating the DNA or RNA preparations. Because plastids are not inherited in *E. chlorotica*, eggs are a source of animal DNA and RNA that is theoretically free of algal contamination (Green et al., 2000). As further PCR controls, primers complementary to the *V. litorea* internal transcribed spacer region (*ITS*1) were used as a positive algal nuclear control (Green et al., 2000), negative control templates (pufferfish and *Dictyostelium* DNA) were tested, and identical PCR reactions were carried out on sea slugs collected from two sites on multiple occasions over a 3-year period (Rumpho et al., 2008; Rumpho et al., 2009). Despite all of these safeguards, one still has to consider the possibility of contaminants when doing any PCR and also the possibility of a 'stray' algal nucleus or another ecto- or endosymbiont making its way into the sea slug and avoiding detection by microscopic and molecular approaches. This further emphasizes the need for high-throughput genome studies to provide direct, definitive evidence for HGT by showing integration into sea slug chromosomal DNA, as elegantly shown for the fungal genes in the pea aphid chromosomes (Moran and Jarvik, 2010).

Preliminary results of transcriptome analyses in kleptoplastic *E. chlorotica*

We have initiated gene discovery in the sea slug using transcriptomic methods to test the HGT hypothesis and to better understand how the kleptoplastic association is formed and sustained. The size of the *E. chlorotica* genome was estimated in collaboration with Gregory (University of Guelph, Canada) to be *ca.* 2.4 Gb. In our approach, RNA was isolated and normalized from adult sea slugs; i.e. that were actively photosynthesizing. Using 175 Mb of data derived from Roche '454' pyrosequencing, we generated a unigene set of 13,941 cDNAs using the Roche GS Assembler V2.3 Software (454 Life Sciences, Branford, CT, USA). The contigs encompassed ca. 10.3 Mb of sequence data with an average size of 739 bp. These data were used in a blastx analysis against a comprehensive genome database (see Moustafa et al., 2009) to identify the top hits and, subsequently, their taxonomic affiliation and reading frame. The predicted reading frame was used to translate the '454' expressed sequence tags when significant (*e*-value <1E–10) hits were found, and the encoded proteins were used for phylogenomic analyses, returning 4219 trees (Moustafa and Bhattacharya, 2008; Moustafa et al., 2008a; Moustafa and Bhattacharya, 2008b). Analyses of these trees underlined the fact that most expressed genes in the adult sea slug are of metazoan derivation and failed to turn up a single robust example of a foreign expressed gene in the adult sea slug partial transcriptome. Similar results were recently reported (Wägele et al., 2010) for two other kleptoplastic sea slugs, *E. timida* and *Plakobranchus ocellatus*, based on partial transcriptome analysis. We did find 32 unigenes that are *Vaucheria* plastid DNA, but they appear to be products of artifactual plastid RNA priming that nonetheless provides clear evidence of plastid photosynthetic activity in the adult starved sea slug.

The surprising absence of algal-derived nuclear-encoded genes involved in photosynthesis remains to be explained and awaits a more exhaustive analysis of the sea slug transcriptome using the Illumina platform (currently underway in the laboratories of M.E.R. and D.B.). However, even with these preliminary transcriptome data, it is apparent that the majority of the greater than 1000 nuclear-encoded plastid-proteins supporting plastid function are not highly expressed in the adult sea slug, and therefore may not be present in the animal nuclear genome. These surprising data led to an alternative hypothesis to the prevailing HGT model, that long-term, functional kleptoplasty in *E. chlorotica* may result from a combination of yet-to-be characterized physical and molecular mechanisms; more specifically, unusual plastid stability (Green et al., 2005) combined with some degree of HGT, and long-term maintenance of cryptic algal products (DNA, RNA and proteins) that persist months after feeding on prey has ended. It should be stressed that additional sea slug transcriptome and genome data are needed to assess these intriguing ideas to explain maintenance of photosynthesis in the sea slug.

Conclusions

Symbiosis has contributed greatly to generating biological diversity and novel functions, including photosynthesis in animals. The endosymbiotic association of algal plastids in the digestive cells of the sea slug *E. chlorotica* provides a rare opportunity to examine how genetic and biochemical components from two distantly related taxa have evolved to form a functional and productive photosynthetic union. Using genomic, biochemical, molecular and cellular approaches we are in the process of unraveling not only how this kleptoplastic association forms, but also how plastids and photosynthesis are sustained for months, and the potential for the evolution of permanent photosynthesis in an animal.

Acknowledgements

This research was supported by the National Science Foundation (grant IOS-0726178 to M.E.R.) and the National Institutes of Health (grant R01ES013679 to D.B.). This is Maine Agricultural and Forest Experiment Station Publication Number 3137, Hatch Project no. ME08361-08MRF (NC 1168). Deposited in PMC for release after 12 months.

References

Adam, Z. and Clarke, A. K. (2002). Cutting edge of chloroplast proteolysis. *Trends Plant Sci.* **7**, 451-456.

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S. et al. (2005). The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.* **52**, 399-451.

Akiba, T., Koyama, K., Ishiki, Y., Kimura, S. and Fukushima, T. (1960). On the mechanism of the development of multiple-drug-resistant clones of *Shigella*. *Jpn. J. Microbiol.* **4**, 219-227.

Andersen, R. A. (2004). Biology and systematics of heterokont and haptophyte algae. *Am. J. Bot.* **91**, 1508-1522.

Andersson, J. O. (2005). Lateral gene transfer in eukaryotes. *Cell. Mol. Life Sci.* **62**, 1182-1197.

Archibald, J. M., Rogers, M. B., Toop, M., Ishida, K. and Keeling, P. J. (2003). Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigelowiella natans*. *Proc. Natl. Acad. Sci. USA* **100**, 7678-7683.

Armbrust, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H., Zhou, S. G., Allen, A. E., Apt, K. E., Bechner, M. et al. (2004). The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* **306**, 79-86.

Aro, E., Virgin, I. and Andersson, B. (1993). Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim. Biophys. Acta* **1143**, 113-134.

Aro, E. M., Suorsa, M., Rokka, A., Allahverdiyeva, Y., Paakkarinen, V., Saleem, A., Battchikova, N. and Rintamaki, E. (2005). Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. *J. Exp. Bot.* **56**, 347-356.

Beagley, C. T., Okada, N. A. and Wolstenholme, D. R. (1996). Two mitochondrial group I introns in a metazoan, the sea anemone *Metridium senile*: one intron contains genes for subunits 1 and 3 of NADH dehydrogenase. *Proc. Natl. Acad. Sci. USA* **93**, 5619-5623.

Bhattacharya, D. and Medlin, L. (1995). The phylogeny of plastids: A review based on comparisons of small subunit ribosomal RNA coding regions. *J. Phycol.* **31**, 489-498.

Bhattacharya, D. and Nosenko, T. (2008). Endosymbiotic and horizontal gene transfer in chromalveolates. *J. Phycol.* **44,** 7-10.

Bhattacharya, D., Yoon, H. W. and Hackett, J. D. (2004). Photosynthetic eukaryotes unite: endosymbiosis connects the dots. *BioEssays* **26**, 50-60.

Biehl, A., Richly, E., Noutsos, C., Salamini, F. and Leister, D. (2005). Analysis of 101 nuclear transcriptomes reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. *Gene* **344**, 33-41.

Bock, R. (2010). The give-and-take of DNA: horizontal gene transfer in plants. *Trends Plant Sci.* **15**, 11-22.

Bock, R. and Timmis, J. N. (2008). Reconstructing evolution: gene transfer from plastids to the nucleus. *BioEssays* **30**, 556-566.

Bowler, C., Allen, A. E., Badger, J. H., Grimwood, J., Jabbari, K., Kuo, A., Maheswari, U., Martens, C., Maumus, F., Otillar, R. P. et al. (2008). The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* **456**, 239-244.

Brandt, K. (1883). Über die morphologische und physiologische bedeutung des chlorophylls bei tieren. *Mit. Zool. Stn. Neapel.* **4**, 191-302.

Carlsson, J., Lagercrantz, U., Sundstrorm, J., Teixeira, R., Wellmer, F., Meyerowitz, E. M. and Glimelius, K. (2007). Microarray analysis reveals altered expression of a large number of nuclear genes in developing cytoplasmic male sterile *Brassica napus* flowers. *Plant J.* **49**, 452-462.

Clark, K. B. and Busacca, M. (1978). Feeding specificity and chloroplast retention in 4 tropical ascoglossa, with a discussion of the extent of chloroplast symbiosis and the evolution of the order. *J. Moll. Stud.* **44**, 272-282.

Clark, K. B., Jensen, K. R. and Stirts, H. M. (1990). Survey for functional kleptoplasty among West Atlantic Ascoglossa (=Sacoglossa) (Mollusca, Opisthobranchia). *Veliger* **33**, 339-345.

de la Cruz, F. and Davies, J. (2000). Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol.* **8**, 128-133.

De Negri, A. and De Negri, G. (1876). Farbstoff aus *Elysia viridis*. *Ber. Deut. Chem. Gesellsch.* **9**, 84.

Erwin, P. M. and Thacker, R. W. (2008). Phototrophic nutrition and symbiont diversity of two Caribbean sponge–cyanobacteria symbioses**.** *Mar. Ecol. Prog. Ser.* **362**, 139-147.

Falkowski, P. G., Katz, M. E., Knoll, A. H., Quigg, A., Raven, J. A., Schofield, O. and Taylor, F. J. R. (2004). The evolution of modern eukaryotic phytoplankton. *Science* **305**, 354-360.

Friesen, T. L., Stukenbrock, E. H., Liu, Z. H., Meinhardt, S., Ling, H., Faris, J. D., Rasmussen, J. B., Solomon, P. S., McDonald, B. A. and Oliver, R. P. (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. *Nat. Genet.* **38**, 953-956.

Gelvin, S. B. (2009). Agrobacterium in the genomics age. *Plant Physiol.* **150**, 1665-1676.

Gibbs, S. P. (1993). The evolution of algal chloroplasts. In *Origins of Plastids* (ed. R. A. Lewin), pp. 107-121. New York: Chapman and Hall.

Gibson, G. D., Toews, D. P. and Bleakney, J. S. (1986). Oxygen production and consumption in the sacoglossan (=ascoglossan) *Elysia chlorotica* Gould. *Veliger* **28**, 397-400.

Gladyshev, E. and Meselson, M. (2008). Extreme resistance of bdelloid rotifers to ionizing radiation. *Proc. Natl. Acad. Sci. USA* **105**, 5139-5144.

Graves, D. A., Gibson, M. A. and Bleakney, J. S. (1979). Digestive diverticula of *Alderia modesta* and *Elysia chlorotica* (Opisthobranchia, Sacoglossa). *Veliger* **21**, 415-422.

Gray, M., Burger, G. and Lang, B. F. (2001). The origin and early evolution of mitochondria. *Genome Biol. Rev.* **2**, 1018.1-1018.5.

Green, B. J., Li, W.-Y., Manhart, J. R., Fox, T. C., Summer, E. J., Kennedy, R. A., Pierce, S. K. and Rumpho, M. E. (2000). Mollusc–algal chloroplast endosymbiosis: photosynthesis, thylakoid protein maintenance, and chloroplast gene expression continue for many months in the absence of the algal nucleus. *Plant Physiol.* **124**, 331-342.

Green, B. J., Fox, T. C., Manhart, J. R. and Rumpho, M. E. (2005). Stability of isolated chromophytic algal chloroplasts that participate in a unique molluscan/algal endosymbiosis. *Symbiosis* **40**, 31-40.

Greene, R. W. and Muscatine, L. (1972). Symbiosis in sacoglossan opisthobranchs – photosynthetic products of animal–chloroplast associations. *Mar. Biol.* **14**, 253-259.

Händeler, K., Grzymbowski, Y. P., Krug, P. K. and Wagele, H. (2009). Functional chloroplasts in metazoan cells – a unique evolutionary strategy in animal life. *Front. Zool.* **6**, 1-18.

Heinemann, J. A. and Kurenbach, B. (2009). Horizontal transfer of genes between microorganisms. In *Encyclopedia of Microbiology,* 3rd edn (ed. M. Schaechter). New York: Academic Press.

Hinde, R. (1980). Chloroplasts "symbiosis" in sacoglossan molluscs. In *Endocytobiology: Endosymbiosis and Cell Biology, a Synthesis of Recent Research* (ed. W. Schwemmler and H. E. A. Schenk), pp. 729-736. Berlin: Walter de Gruyter.

Hinde, R. and Smith, D. C. (1972). Persistence of functional chloroplast in *Elysia viridis* (Opisthobranchia, Sacoglossa). *Nat. New Biol.* **239**, 30-31.

Hinde, R. and Smith, D. C. (1974). "Chloroplast symbiosis" and extent to which it occurs in Sacoglossa (Gastropoda, Mollusca). *Biol. J. Linn. Soc.* **6**, 349-356.

Hooge, M. D. and Tyler, S. (2008). Acoela (Acoelomorpha) from Bocas del Toro, Panama. *Zootaxa* **1719**, 1-40.

Jensen, K. R. (1986). Observations on copulation in two species of Elysia from Florida (Opisthobranchi, Ascoglossa). *Ophelia* **25**, 25-32.

Kawaguti, S. and Yamasu, T. (1965). Electron microscopy on the symbiosis between an elysioid gastropod and chloroplasts from a green alga. *Biol. J. Okayama Univ.* **II**, 57-64.

Keeling, P. J. (2004). Diversity and evolutionary history of plastids and their hosts. *Am. J. Bot.* **91**, 1481-1493.

Keeling, P. J. (2009). Functional and ecological impacts of horizontal gene transfer in eukaryotes. *Curr. Opin. Genet. Dev.* **19**, 613-619.

Klasson, L., Kambris, Z., Cook, P. E., Walker, T. and Sinkins, S. P. (2009). Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. *BMC Genomics* **10**, 33.

Kondo, N., Nikoh, N., Ijichi, N., Shimada, M. and Fukatsu, T. (2002). Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. *Proc. Natl. Acad. Sci. USA* **99**, 14280-14285.

Koonin, E. V., Makarova, K. S. and Aravind, L. (2001). Horizontal gene transfer in prokaryotes: quantification and classification. *Annu. Rev. Microbiol.* **55**, 709-742.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001). Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921.

Lane, C. E. and Archibald, J. M. (2008). The eukaryotic tree of life: endosymbiosis takes its TOL. *Trends Ecol. Evol.* **23**, 268-275.

Lavrov, D. V. (2007). Key transitions in animal evolution: a mitochondrial DNA perspective. *Integr. Comp. Biol.* **47**, 734-743.

Lee, R. E. (2008). Heterokontophyta, Xanthophyceae. In *Phycology* (ed. R. E. Lee), pp. 413-423. Cambridge: Cambridge University Press.

Loftus, B., Anderson, I., Davies, R., Alsmark, U. C. M., Samuelson, J., Amedeo, P., Roncaglia, P., Berriman, M., Hirt, R. P., Mann, B. J. et al. (2005). The genome of the protist parasite *Entamoeba histolytica*. *Nature* **433**, 865-868.

Mann, N. H., Cook, A., Millard, A., Bailey, S. and Clokie, M. (2003). Marine ecosystems: bacterial photosynthesis genes in a virus. *Nature* **424**, 741.

Marchetti, A., Parker, M. S., Moccia, L. P., Lin, E. O., Arrieta, A. L., Ribalet, F., Murphy, M. E., Maldonado, M. T. and Armbrust, E. V. (2009). Ferritin is used for iron storage in bloom-forming marine pennate diatoms. *Nature* **457**, 467-470.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, M. and Penny, D. (2002). Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. USA* **99**, 12246-12251.

McFadden, G. I. (2001). Primary and secondary endosymbiosis and the origin of plastids. *J. Phycol.* **37**, 951-959.

Meeks, J. C., Elhai, J., Thiel, T., Potts, M., Larimer, F., Lamerdin, J., Predki, P. and Atlas, R. (2001). An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. *Photosyn. Res.* **70**, 85-106.

Moran, N. A. and Jarvik, T. (2010). Lateral transfer of genes from fungi underlies carotenoids production in aphids. *Science* **328**, 624-627.

Moustafa, A. and Bhattacharya, D. (2008). PhyloSort: a user-friendly phylogenetic sorting tool and its application to estimating the cyanobacterial contribution to the nuclear genome of *Chlamydomonas*. *BMC Evol. Biol.* **8**, 6.

Moustafa, A., Chan, C., Danforth, M., Zear, D., Ahmed, H., Jadhav, N., Savage, T. and Bhattacharya, D. (2008a). A phylogenomic approach for studying plastid endosymbiosis. *Gen. Inform. Intern. Conf. Genome Inform.* **21**, 165-176.

Moustafa, A., Reyes-Prieto, A. and Bhattacharya, D. (2008b). Chlamydiae has contributed at least 55 genes to Plantae with predominantly plastid functions. *PLoS ONE* **3**, e2205.

Moustafa, A., Beszteri, B., Maier, U. G., Bowler, C., Valentin, K. and Bhattacharya, D. (2009). Genomic footprints of a cryptic plastid endosymbiosis in diatoms. *Science* **324**, 1724-1726.

Muscatine, L., Pool, R. R. and Trech, R. K. (1975). Symbiosis of algae and invertebrates – aspects of symbiont surface and host-symbiont interface. *Trans. Am. Microsc. Soc.* **94**, 450-469.

Nelson, N. and Yocum, C. F. (2006). Structure and function of photosystems I and II. *Annu. Rev. Plant Biol.* **57**, 521-565.

Nikoh, N., Tanaka, K., Shibata, F., Kondo, N., Hizume, M., Shimada, M. and Fukatsu, T. (2008). *Wolbachia* genome integrated in an insect chromosome:

Evolution and fate of laterally transferred endosymbiont genes. *Gen. Res.* **18**, 272-280.
Norton, J. H., Shepherd, M. A., Long, H. M. and Fitt, W. K. (1992). Zooxanthellal tubular system in the giant clam. *Biol. Bull.* **183**, 503-506.
Nosenko, T. and Bhattacharya, D. (2007). Horizontal gene transfer in chromalveolates. *BMC Evol. Biol.* **7**, 173.
Pierce, S. K., Biron, R. W. and Rumpho, M. E. (1996). Endosymbiotic chloroplasts in molluscan cells contain proteins synthesized after plastid capture. *J. Exp. Biol.* **199**, 2323-2330.
Pierce, S. K., Curtis, N. E., Hanten, J. J., Boerner, S. L. and Schwartz, J. A. (2007). Transfer, integration and expression of functional nuclear genes between multicellular species. *Symbiosis* **43**, 57-64.
Pierce, S. K., Curtis, N. E. and Schwartz, J. A. (2009). Chlorophyll a synthesis by an animal using transferred algal nuclear genes. *Symbiosis* **49**, 121-131.
Raghavendra, A. S. (1998). *Photosynthesis. A Comprehensive Treatise*. Cambridge: Cambridge University Press.
Raven, J. A., Beardall, J., Flynn, K. and Maberly, S. (2009). Phagotrophy in the origins of photosynthesis in eukaryotes and as a complementary mode of nutrition in phototrophs: relation to Darwin's insectivorous plants. *J. Exp. Bot.* **60**, 3975-3987.
Reyes-Prieto, A., Weber, A. P. M. and Bhattacharya, D. (2007). The origin and establishment of the plastid in algae and plants. *Annu. Rev. Genet.* **41**, 147-168.
Richards, T. A., Soanes, D. M., Foster, P. G., Leonard, G., Thornton, C. R. and Talbot, N. J. (2009). Phylogenomic analysis demonstrates a pattern of rare and ancient horizontal gene transfer between plants and fungi. *Plant Cell* **21**, 1897-1911.
Richardson, A. O. and Palmer, J. D. (2007). Horizontal gene transfer in plants. *J. Exp. Bot.* **58**, 1-9.
Richly, E. and Leister, D. (2004). An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of *Arabidopsis* and rice. *Gene* **329**, 11-16.
Rocap, G., Larimer, F. W., Lamerdin, J., Malfatti, S., Chain, P., Ahlgren, N. A., Arellano, A., Coleman, M., Hauser, L. and Hess, W. R. (2003). Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature* **424**, 1042-1047.
Roelofs, J. and Van Haastert, P. J. M. (2001). Genomics: genes lost during evolution. *Nature* **411**, 1013-1014.
Rot, C., Goldfarb, I., Ilan, M. and Huchon, D. (2006). Putative cross-kingdom horizontal gene transfer in sponge (Porifera) mitochondria. *BMC Evol. Biol.* **6**, 71.
Rumpho, M. E., Summer, E. J. and Manhart, J. R. (2000). Solar-powered sea slugs. Mollusc/algal chloroplast symbiosis. *Plant Physiol.* **123**, 29-38.
Rumpho, M. E., Summer, E. J., Green, B. J., Fox, T. C. and Manhart, J. R. (2001). Mollusc/algal chloroplast symbiosis: how can isolated chloroplasts continue to function for months in the cytosol of a sea slug in the absence of an algal nucleus? *Zoology* **104**, 303-312.
Rumpho, M. E., Dastoor, F. P., Manhart, J. R. and Lee, J. (2006). The Kleptoplast. In *Advances in Photosynthesis and Respiration – the Structure and Function of Plastids,* Vol. 23 (ed. R. R. Wise and J. K. Hoober), pp. 451-473. New York: Springer.
Rumpho, M. E., Worful, J. M., Lee, J., Kannan, K., Tyler, M. S., Bhattacharya, D., Moustafa, A. and Manhart, J. R. (2008). Horizontal gene transfer of the algal nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica*. *Proc. Natl. Acad. Sci. USA* **105**, 17867-17871.
Rumpho, M. E., Pochareddy, S., Worful, J. M., Summer, E. J., Bhattacharya, D., Pelletreau, K. N., Tyler, M. S., Lee, J., Manhart, J. R. and Soule, K. M. (2009). Molecular characterization of the Calvin cycle enzyme phosphoribulokinase in the stramenopile alga *Vaucheria litorea* and the plastid hosting mollusc *Elysia chlorotica*. *Mol. Plant* **2**, 1384-1396.
Sakamoto, W. (2006). Protein degradation machineries in plastids. *Annu. Rev. Plant Biol.* **57**, 599-621.
Salzberg, S. L., White, O., Peterson, J. and Eisen, J. A. (2001). Microbial genes in the human genome: lateral transfer or gene loss? *Science* **292**, 1903-1906.
Sánchez-Puerta, M. V. and Delwiche, C. F. (2008). A hypothesis for plastid evolution in chromalveolates. *J. Phycol.* **44**, 1097-1107.
Sanders, I. R. (2006). Rapid disease emergence through horizontal gene transfer between eukaryotes. *Trends Ecol. Evol.* **21**, 656-658.
Schwartz, J. A., Curtis, N. E. and Pierce, S. K. (2010). Using algal transcriptome sequences to identify transferred genes in the sea slug, *Elysia chlorotica*. *Evol. Biol.* **37**, 29-37.
Soule, K. M. (2009). Light-regulated photosynthetic gene expression and enzyme activity in the heterokont alga *Vaucheria litorea* and its symbiotic partner the sacoglossan mollusc *Elysia chlorotica*. MSc thesis. University of Maine.
Starcevic, A., Akthar, S., Dunlap, W. C., Shick, J. M., Hranueli, D., Cullum, J. and Long, P. F. (2008). Enzymes of the shikimic acid pathway encoded in the genome of a basal metazoan, *Nematostella vectensis*, have microbial origins. *Proc. Natl. Acad. Sci. USA* **105**, 2533-2537.
Stegemann, S. and Bock, R. (2009). Exchange of genetic material between cells in plant tissue grafts. *Science* **324**, 649-651.
Sullivan, M. B., Coleman, M. L., Weigele, P., Rohwer, F. and Chisholm, S. W. (2005). Three *Prochlorococcus* cyanophage genomes: signature features and ecological interpretations. *PLoS Biol.* **3**, e144.
Trench, R. K. (1969). Chloroplasts as functional endosymbionts in the mollusc *Tridachnia crispata* (Bërgh), (Opisthobranchia, Sacoglossa). *Nature* **222**, 1071-1072.
Trench, R. K. (1975). Of 'leaves that crawl': functional chloroplasts in animal cells. In *Symposia of the Society for Experimental Biology* (ed. D. H. Jennings), pp. 229-265. London: Cambridge University Press.
Trench, R. K. (1993). Microalgal-invertebrate symbiosis: a review. *Endocyt. Cell Res.* **9**, 135-175.
Trench, R. K., Greene, R. W. and Bystrom, B. G. (1969). Chloroplasts as functional organelles in animal tissues. *J. Cell Biol.* **42**, 404-417.
Trench, R. K., Trench, M. E. and Muscatine, L. (1972). Symbiotic chloroplasts – their photosynthetic products and contribution to mucus synthesis in two marine slugs. *Biol. Bull.* **142**, 335-349.
Trench, R. K., Boyle, E. J. and Smith, D. C. (1973a). The association between chloroplasts of *Codium fragile* and the mollusc *Elysia viridis*. I. Characteristics of isolated *Codium* chloroplasts. *Proc. R. Soc. Lond. B Biol. Sci.* **184**, 51-61.
Trench, R. K., Boyle, E. J. and Smith, D. C. (1973b). Association between chloroplasts of *Codium fragile* and the mollusc *Elysia viridis*. II. Chloroplast ultrastructure and photosynthetic carbon fixation in *Elysia viridis*. *Proc. R. Soc. Lond.* **184**, 63-81.
van Oppen, M. J. H., Catmull, J., McDonald, B. J., Hislop, N. R., Hagerman, P. J. and Miller, D. J. (2002). The mitochondrial genome of *Acropora tenuis* (Cnidaria: Scleractinia) contains a large group I intron and a candidate control region. *J. Mol. Evol.* **55**, 1-13.
Venn, A. A., Loram, J. E. and Dougalas, A. E. (2008). Photosynthetic symbiosis in animals. *J. Exp. Bot.* **59**, 1069-1080.
Voolstra, C. R., Schwarz, J. A., Schnetzer, J., Sunagawa, S., Desalvo, M. K., Szmant, A. M., Coffroth, M. A. and Medina, M. (2009). The host transcriptome remains unaltered during the establishment of coral–algal symbioses. *Mol. Ecol.* **1**, 1823-1833.
Wägele, H., Deusch, O., Händeler, K., Martin, R., Schmitt, V., Christa, G., Pinzger, B., Gould, S. B., Dagan, T., Klussmann-Kolb, A. and Martin, W. (2010). Transcriptomic evidence that longevity of acquired plastids in the photosynthetic slugs *Elysia timida* and *Plakobrachus ocellatus* does not entail lateral transfer of algal nuclear genes. *Mol. Biol. Evol.* doi:10.1093/molbev/msq239.
Wasmuth, J., Schmid, R., Hedley, A. and Blaxter, M. (2008). On the extent and origins of genetic novelty in the phylum nematoda. *PLoS Negl. Trop. Dis.* **2**, e258.
West, H. H. (1979). Chloroplast symbiosis and development of the ascoglossan opistobranch *Elysia chlorotica*. PhD thesis, Northeastern University, Boston, MA.
West, H. H., Harrigan, J. and Pierce, S. K. (1984). Hybridization of two populations of a marine opistobranch with different development patterns. *Veliger* **26**, 199-206.
Willerslev, E., Mourier, T., Hansen, A. J., Christensen, B., Barnes, I. and Salzberg, S. L. (2002). Contamination in the draft of the human genome masquerades as lateral gene transfer. *DNA Seq.* **13**, 75-76.
Williamson, S. J., Rusch, D. B., Yooseph, S., Halpern, A. L., Heidelberg, K. B., Glass, J. I., Andrews-Pfannkoch, C., Fadrosh, D., Miller, C. S., Sutton, G. et al. (2008). The sorcerer II global ocean sampling expedition: metagenomic characterization of viruses within aquatic microbial samples. *PLoS ONE* **3**, e1456.
Woolfit, M., Iturbe-Ormaetxe, I., McGraw, E. A. and O'Neill, S. L. (2009). An ancient horizontal gene transfer between mosquito and the endosymbiotic bacterium *Wolbachia pipientis*. *Mol. Biol. Evol.* **26**, 367-374.
Yellowlees, D., Rees, T. and Leggat, W. (2008). Metabolic interactions between algal symbionts and invertebrate hosts. *Plant Cell Environ.* **31**, 679-694.
Yoon, H. S., Hackett, J. D., Van Dolah, F. M., Nosenko, T., Lidie, K. L. and Bhattacharya, D. (2005). Tertiary endosymbiosis driven genome evolution in dinoflagellate algae. *Mol. Biol. Evol.* **22**, 1299-1308.