

Solar-Powered Sea Slugs. Mollusc/Algal Chloroplast Symbiosis^{1,2}

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Solar-powered “leaves that crawl”? This description of photosynthetic sea slugs (adapted from Bill Rudman [www.austmus.gov.au/seaslugs/solarpow.htm] and Robert Trench [1975]) aptly describes the symbiotic association that occurs between certain molluscan sea slugs and algal chloroplasts. Faced with life without a protective shell in a predatory environment, some sea slugs evolved a protective mechanism dependent largely upon camouflage provided by symbiont plastids (Fig. 1). Sea slugs in the opisthobranch order of Gastropods, Ascoglossa (= Sacoglossa), have taken this one step further. They feed by slicing or puncturing siphonaceous algal cells and sucking out the cell contents. All of the contents, including the algal nucleus, are discarded except for the chloroplasts, which are engulfed phagocytotically into the digestive cells (see micrographs in Fig. 2, A and B). By distributing the “photosynthetic factories” throughout their extensively branched digestive system just one cell layer beneath the epidermis, the sea slugs not only blend into the green algal bed (Fig. 1C), but also capture light energy to fuel photoautotrophic CO₂ fixation (Fig. 3). In some cases, the resulting carbon products can totally sustain the sea slugs for several months in the absence of an algal food source (for review, see Trench, 1975; Mujer et al., 1996), and also serve as precursors for the synthesis of chemical defense compounds and the copious mucus that bathes and protects these animals (Paul and Van Alstyne, 1988).

Symbiotic associations between organisms, even of different kingdoms, is not that unusual. However, in almost all cases the association is between two intact, free-living organisms, both of which have retained their complete cellular genetic makeup (Trench, 1975; Douglas, 1994). Such associations are typically inter-

cellular; however, if they are intracellular, the symbiont is frequently isolated from the host’s cytosol by sequestration in a vacuole or a host-provided membrane. What makes the sea slug/algal chloroplast symbiosis so remarkable is that the symbiont is a “naked,” foreign organelle sustained intracellularly in direct contact with the host sea slug cytosol (Fig. 2, A and B), and the symbiont remains functional for several months despite the absence of any algal nucleo-cytosolic influence. In the most remarkable case yet reported, the symbiotic association between the sea slug *Elysia chlorotica* Gould and chloroplasts from the chromophytic alga *Vaucheria litorea* C. Agardh, the symbionts remain intact and functional for at least 9 months (Mujer et al., 1996; Pierce et al., 1996). This degree of activity is highly unusual, given the overwhelming data on the importance of nuclear-encoded proteins for essentially every plastid function. Even the largest chloroplast genomes identified to date code for less than 25% of the gene products necessary for plastid function (Reith and Munholland, 1995; Martin and Herrmann, 1998).

In this *Update*, we provide a short review of some of the symbiotic associations between algal chloroplasts and ascoglossan molluscs and describe the varying longevity and functional capacity of the symbionts. We will also address the following questions: How can an isolated organelle, normally dependent upon its own nucleo-cytosol for activity and survival, remain physically stable and function for months in a foreign cell? Are we seeing tertiary endosymbiosis in action? Has lateral gene transfer occurred between a eukaryotic alga and an animal nucleus?

WHAT CONSTITUTES A SYMBIOTIC ASSOCIATION?

There is some disagreement on whether an association between an organism and an isolated organelle such as a chloroplast constitutes symbiosis, since the symbiont (chloroplast) is not a free-living organism. The term symbiosis was first defined as “unlike organisms living together.” “Unlike organisms” came to mean different species, and symbiosis therefore changed to reflect “prolonged physical associations without respect to outcome.” In the early 1900s, the Russian scientist K.S. Mereschkovsky proposed that

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² The phrase “solar-powered sea slugs” is used with the permission of Dr. Bill Rudman, Australian Museum, and the Sea Slug Forum, <http://www.austmus.gov.au/seaslugs/solarpow.htm>.

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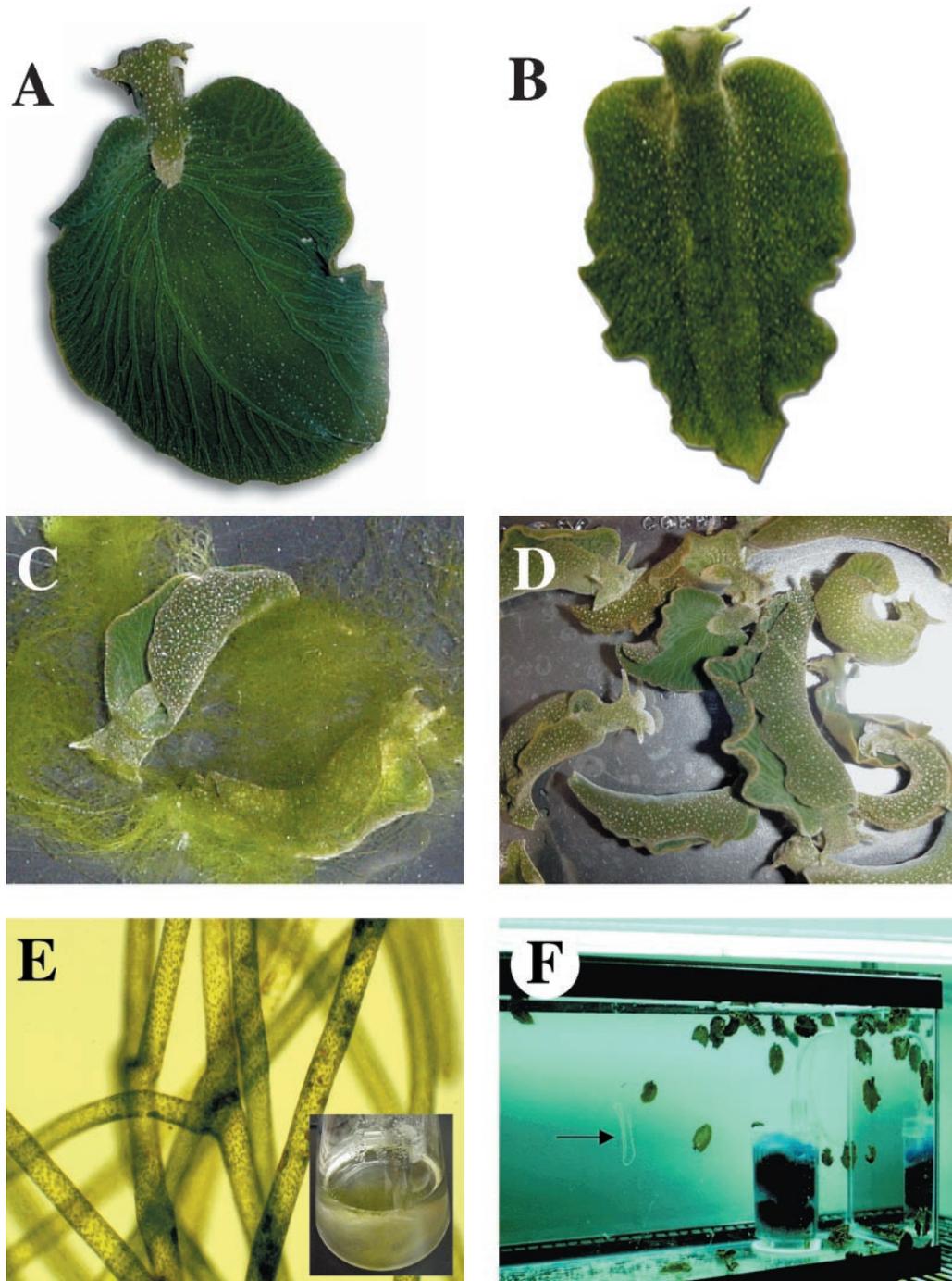


Figure 1. A, Dorsal view of the mollusc *E. chlorotica* illustrating the distribution of green pigment (chloroplasts), giving *E. chlorotica* a uniform green appearance. The chloroplasts are found in cells lining the extensive digestive diverticula, which ramify throughout the body. Animals are typically found in nature as small as 1 or 2 cm to as large as 6 cm, as shown here. B, Ventral view of *E. chlorotica* illustrating the uniform dispersal of chloroplasts and the recessed, sucking mouth. C, Two camouflaged *E. chlorotica* specimens feeding on *V. litorea*. D, Several specimens of *E. chlorotica* showing the variation in size and body form. When left undisturbed in strong light, the sea slugs will unfold their parapodia (lateral folds) and “sunbathe.” Any slight movement or even a shadow will cause them to fold up and exude mucus. E, Isolated *V. litorea* filaments (about 1–2 mm in diameter) and filaments growing in a culture flask (inset). The alga can be easily cultured in one-quarter-strength seawater and exhibits rapid growth with hair-like filaments extending vertically through the media. F, Sea slugs are easily cultured in aquaria containing full-strength artificial seawater, overhead lighting, and maintained at 10°C. The animals will move slowly along the walls of the aquarium, unfolding their parapodia presumably to increase the body surface area exposed to the overhead light. Non-pigmented eggs are produced in a mucus mass and deposited on the aquaria walls (see arrow). The eggs serve as a source of pure animal DNA, since no plastids or plastid DNA are found in them.

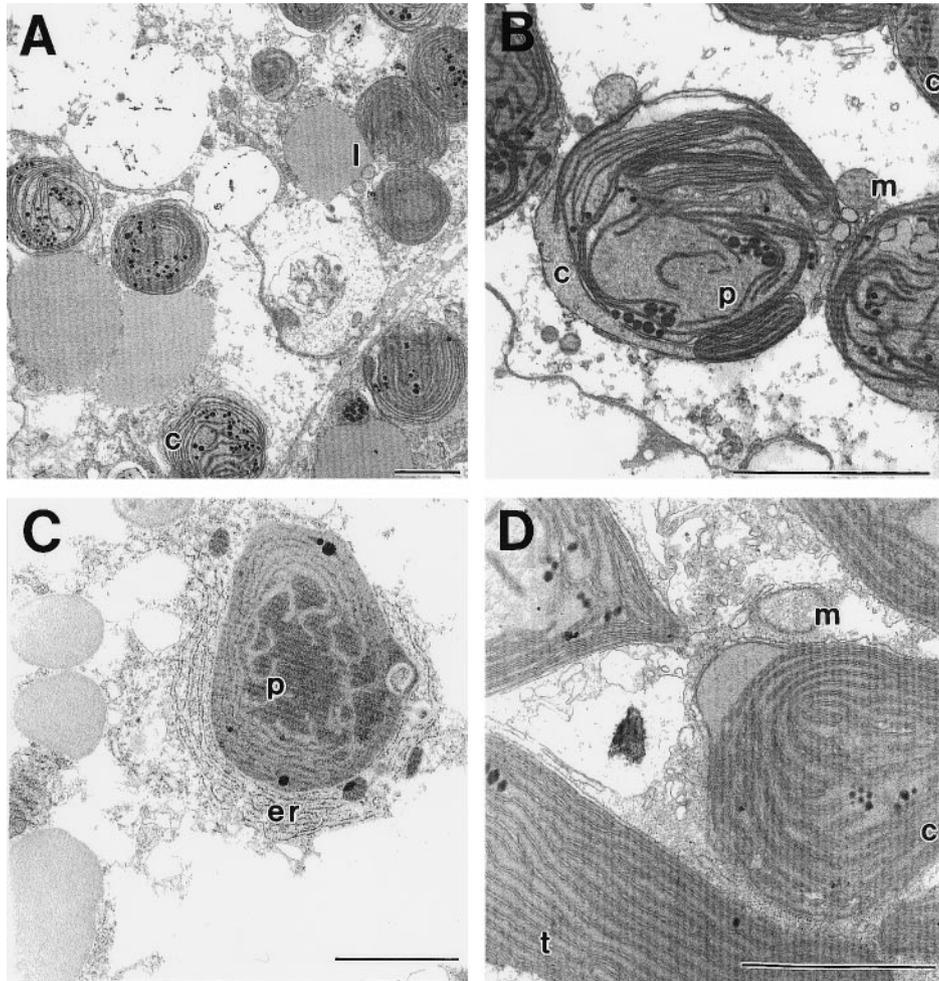


Figure 2. Electron micrographs of *E. chlorotica* (A and B) and *V. litorea* (C and D). A and B, Symbiont chloroplasts (c) are maintained intracellularly in a specific cell layer lining the digestive tract of *E. chlorotica*. The intact chloroplast envelope is evident in B, and no chloroplast endoplasmic reticulum (er) is associated with the symbionts. Large pyrenoids (p), lipid accumulation (l), mitochondria (m), and unstacked chloroplast tri-lamellar thylakoids (t) are evident in both sea slug (A and B) and algal plastids (C and D). C and D, *V. litorea* is a siphonaceous, chromophytic alga with numerous chloroplasts in the thin cytoplasm lining the sides of the filaments. Chloroplast endoplasmic reticulum is only seen associated with plastids in *V. litorea*. The association is loose and the number of layers varies. Scale bars = 5 μm . (For complete electron microscopy methods, see Mujer et al., 1996.)

chloroplasts originated from blue-green algae (cyanobacteria), a process he named symbiogenesis or “the origin of evolutionary novelty via symbiosis” (for review, see Margulis, 1990). Trench (1975) defined intracellular symbiosis as “the coexistence of at least two genomes of divergent evolutionary origins occupying the same cytoplasmic environment.” In a review of several symbiotic associations, Douglas (1994) emphasized that symbiosis is not dependent on mutual benefit to the partners, but rather that at least one of the partners acquires a new metabolic property. Considering both Trench’s and Douglas’ definitions, we conclude that the intracellular association of algal chloroplasts with molluscan cells can be considered a unique symbiotic association. The chloroplast represents a symbiont genome, and the host mollusc acquires a new metabolic capability, photosynthesis.

Others prefer to use the term “kleptoplasty” or “something borrowed” to describe the chloroplast symbiosis (Waugh and Clark, 1986; Clark et al., 1990). Regardless of the definition or term used, today it is universally recognized that great biological novelty and diversity come from symbiotic associations, and that symbiosis is a widespread biological phenomenon.

GREEN SEA SLUGS HAVE BEEN OBJECTS OF CURIOSITY SINCE THE 1800s

The presence of a green pigment in sea slugs was first reported in 1876 by De Negri and De Negri in *Elysia viridis*, followed by the isolation of small green “organisms” from the animal by Brandt (1883). Interest in these early studies was renewed following the detailed report by Kawaguti and Yamasu in 1965

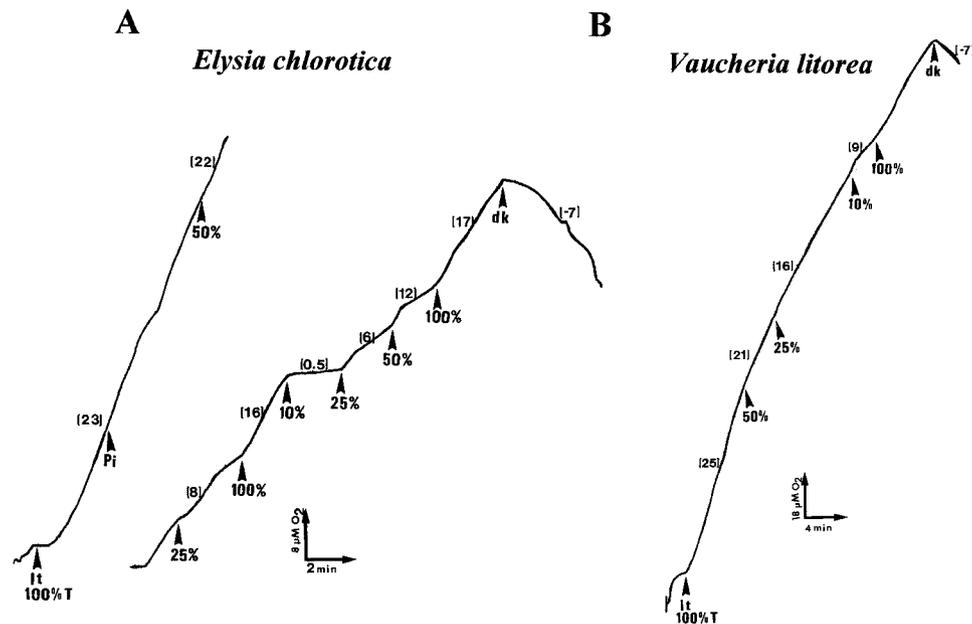


Figure 3. Light- and CO₂-dependent oxygen evolution by *E. chlorotica* and *V. litorea*. The pigmented sea slugs display oxygen evolution that is dependent upon both light and CO₂. Oxygen exchange by intact animals or algal filaments was measured at 15°C using a DW1 electrode (Hansatech, King's Lynn, UK) with 100% T = PAR of 1,500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The numbers in parentheses reflect oxygen evolution rates ($\mu\text{mol O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$) as influenced by light intensity. Maximal measurable rates vary depending upon the size (thickness) of the sea slugs, their willingness to open up, and the growth conditions of the algae. Rates as high as 30 and 120 $\mu\text{mol O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ have been measured for *E. chlorotica* and *V. litorea*, respectively. Pi, Inorganic phosphate; dk, dark; lt, light.

demonstrating the presence of algal chloroplasts, not unicellular algae, in animal cells. Electron microscopy revealed that the green structures housed in the digestive cells of *Elysia atroviridis* were structurally identical to the chloroplasts in the green alga *Codium fragile*, upon which the sea slug was observed to feed. Metabolic function of the plastids was inferred, but not measured. Since Kawaguti and Yamasu's report (1965), the natural curiosity surrounding these animals has intrigued many scientists, leading to several pioneering studies by Trench (1975), Taylor (1970), Greene (1974), and Muscatine et al. (1975) in the late 1960s and 1970s. Due mostly to lack of federal support (Margulis, 1990), studies on these symbiotic organisms stalled in the 1980s and early 1990s. However, with increased recognition of the global role of symbiosis in eukaryotic cellular evolution, accompanied by recent advances in molecular biology and the development of the technical means to handle the copious mucus produced by the animals (which interferes with almost all experimental procedures), research in this area has again been renewed and supported at the federal level.

DISTRIBUTION OF SEA SLUG/ALGAL CHLOROPLAST SYMBIOSES

The majority of sea slugs that form chloroplast symbioses belong to the molluscan order Ascoglossa (class Gastropoda, subclass Opisthobranchia). A sur-

vey of 86 ascoglossan species revealed that 82% are primarily green in color, indicating the widespread nature of pigment/plastid retention in this order. The identity of the algal food source of many of these associations is unknown (Greene, 1974). Chloroplast retention among this group varies from non-retention (i.e. they feed on the algae, but do not acquire plastids) to long-term functional retention (photosynthetic activity beyond 1 week in "starved" animals, i.e. cultured in the absence of algae; Clark et al., 1990). The most primitive retention of plastids (non-functional) has been observed in shelled ascoglossans, whereas functional retention is common in the elysiacan families and to a lesser extent in the stiligeroid (cerata bearing) families. Biochemical activity reported for various functional sea slug/plastid combinations includes: light- and CO₂-dependent O₂ evolution, CO₂ fixation, export of photosynthate, carotenoid but not chlorophyll biosynthesis, and the metabolism of photosynthate into mucus production (for review, see Trench, 1975). Chloramphenicol-sensitive chloroplast protein synthesis in *E. viridis* was inferred by electron microscopy autoradiography of [³H]Leu-labeled cells (Trench, 1975). However, specific incorporation into Rubisco was not detected in either *E. viridis* or *Elysia crispata*, leading to the conclusion that Rubisco has a reduced turnover rate in these organisms. Reviews by Taylor (1970), Hinde and Smith (1974), Trench (1975), and Clark et al. (1990) detail studies from the

1960s through the 1980s on chloroplast symbiosis in the ascoglossans and the extent of their functionality.

WHAT MAKES A GOOD CHLOROPLAST DONOR?

It was originally believed that all elysioid symbionts originated from the green algal order Siphonales. We now know, however, that the spectrum of algal donors is much broader (for review, see Clark et al., 1990). Regardless of taxonomic origin, siphonaceous organization and succulent filaments appear to be the most important attributes facilitating feeding. Similar to the chlorophyte or green algal symbiont providers, the chromophyte *V. litorea* exhibits a siphonaceous morphology (Hibberd, 1980; Fig. 1E). The filaments have few distinct cross walls and the center is occupied by a large vacuole with a thin layer of multinucleate cytoplasm containing numerous chloroplasts. Therefore, sea slugs can extract a large number of chloroplasts with minimal perturbation simply by puncturing the thin cell wall with their specially adapted radular tooth and sucking out the contents (Jensen, 1993). This ease of obtaining chloroplasts is probably one reason why siphonaceous algae are preferred plastid donors for the endosymbiotic process. Jensen (1993) compared the radular teeth morphology of 55 species within 21 genera of ascoglossans. Widespread morphological adaptations of the feeding apparatus for suctorial feeding were observed in this group of herbivores, with cell wall structure of the algal diet being the major determining factor.

Species of *Elysia* that feed on coenocytic *Caulerpa* do so only when the tissue is young and succulent before the rigid thalli develop (Clark and Busacca, 1978). *Elysia timida*'s survival is dependent upon adapting to the life cycle of the chlorophycean alga *Acetabularia acetabulum* (Marín and Ros, 1992). The sea slugs graze on the alga and store the plastids moving along the stalk away from high calcified areas. *Elysia tuca* further adjusts to stalk calcification and will feed on the non-calcified gametangic thalli of *Halimeda incrassata* (Clark and DeFreese, 1987). Retention or storage of plastids may serve as an energy insurance policy against algal calcification and/or the scarcity of algae, both of which would prevent food acquisition. In temperate species, the ability to overwinter with plastids would provide the sea slugs with an early energy reserve in the spring to sustain them while they search for food, which is scarce before the marsh warms and supports algal growth.

E. CHLOROTICA/V. LITOREA SYMBIOSIS

The symbiotic association between *E. chlorotica* and *V. litorea* is the longest-lived and one of the most thoroughly characterized at the biochemical and molecular levels (Mujer et al., 1996; Pierce et al., 1996;

Green et al., 2000). Graves et al. (1979) were the first to demonstrate that *E. chlorotica* contains plastids derived from the Xanthophyte *V. litorea*. They observed that the chloroplasts did not accumulate in the lumen of the digestive diverticula (which would be an indication of digestion), but instead were found distributed throughout the cell. The plastids were generally structurally intact, with well defined trilamellar, unstacked thylakoids, and bounded by two envelope membranes. The outer envelope membrane appeared to be in direct contact with the animal cytoplasm, and no other algal cellular structures were observed in the animal cell. Light- and CO₂-dependent oxygen evolution was observed in *E. chlorotica* (see example in Fig. 3), and an absorption spectra strongly mimicked that of *V. litorea*, suggesting similar pigment profiles between the animal and the algae (Graves et al., 1979). Gibson et al. (1986) reported that healthy, presumably photosynthetic animals could be maintained for at least 4 months in the absence of algal food. Today we know this can extend to at least 9 or 10 months after removal from the marsh (Mujer et al., 1996; Pierce et al., 1996; Green et al., 2000).

E. chlorotica Life Cycle

E. chlorotica is native to saltwater marshes from South Florida to as far north as Nova Scotia (Clark and Busacca, 1978; West, 1979), inhabiting waters varying in salinity from 3‰ to 32‰ (West et al., 1984). They are frequently observed grazing on filaments of *V. litorea*, although they will also feed on *Vaucheria compacta*. West et al. (1984) have demonstrated that it is possible to culture *E. chlorotica* through its complete life cycle as long as the appropriate unicellular alga is maintained for the planktonic veligers, and filamentous *Vaucheria* species are maintained for metamorphosis and chloroplast acquisition by the juvenile sea slugs. The approximately 11-month life cycle of *E. chlorotica* begins with egg laying by the adults in late spring (West, 1979; West et al., 1984; see eggs in Fig. 1F). Planktonic veligers hatch within 7 to 8 d following egg deposition and spend about 14 d feeding on unicellular algae. When provided with filaments of *V. litorea*, metamorphosis of the veligers into juvenile sea slugs occurs within 1 to 2 d. Spontaneous metamorphosis is rare and specificity for *V. litorea* or *V. compacta* is very high. The juvenile sea slugs then feed on the *V. litorea* filaments, acquiring green pigmentation as a result of incorporation of intact chloroplasts into specific cells lining the digestive gland (Fig. 2, A and B). Over the next several months, the sea slugs continue to feed on *V. litorea* if it is available and/or sustain themselves by photoautotrophic CO₂ fixation using their newly acquired chloroplasts. In laboratory culture, the sea slugs are kept apart from the algae (Fig. 1F), thus sustaining themselves totally by photoau-

totrophic CO₂ fixation. Interestingly, shortly after egg production in late spring, mass death of the adults occurs synchronously in the laboratory or in the field. The lifespan is basically the same whether the sea slugs are left in their native marsh environment or cultured in saltwater aquaria and kept apart from all algal food sources (West et al., 1984; M. Rumpho, personal observations). Pierce et al. (1999) have attributed this mass death not to loss of chloroplast function, but to a viral pathogen endemic in the animal population.

Biochemical and Molecular Analyses

E. chlorotica produces and secretes copious amounts of mucopolysaccharides, which contaminate efforts to isolate plastids, nucleic acids, and proteins when utilizing standard protocols. Two technical advances have permitted the study of the endosymbiosis at the biochemical and molecular levels. One was the development of protocols to isolate DNA suitable for Southern analysis (Rumpho et al., 1994). The other was the discovery that a mucolytic agent, *N*-acetyl-L-Cys (Sigma, St. Louis), is effective in ridding enough mucus from the animal to permit protein and plastid isolation (Pierce et al., 1996). Following these advances, it was found that *E. chlorotica* cultured for 8 months in the absence of algae still contained plastid DNA and transcripts of two chloroplast genes, *psbA* and 16S rRNA (Mujer et al., 1996). Several photosystem proteins predicted to be plastid synthesized, D1, D2, and CP43, were also found to be present. Immunoprecipitation of proteins following in vivo radiolabeling of *E. chlorotica* in the presence or absence of chloroplast protein translation inhibitors indicated that at least two proteins, D1 and Rubisco LS, were being actively synthesized in the symbionts for as long as 8 months (Pierce et al., 1996).

Nuclear-Encoded Proteins

Plastid protein synthesis has been demonstrated in *E. chlorotica*, but what about nuclear encoded proteins? Are they being synthesized by the animal and targeted to the symbiont chloroplasts? This has not been conclusively answered yet, but is being actively pursued. Preliminary evidence, obtained by labeling sea slugs with [³⁵S]Met in the presence of chloroplast gene expression inhibitors followed by isolation of the plastids, suggests that several unidentified proteins are synthesized in the cytosol and incorporated into the plastids (Pierce et al., 1996). One nuclear-encoded protein, a light-harvesting complex homolog cross-reactive to fucoxanthin chlorophyll *a/c* binding protein from another chromophyte, *Pavlova gyrans*, was detected in *E. chlorotica*, but incorporation of [³⁵S]Met into this protein has not been verified (Mujer et al., 1996; Pierce et al., 1996).

WHAT SUSTAINS THE LONG-TERM SYMBIONT ACTIVITY?

The simplest explanation for long-term plastid activity in the molluscs is that remnant algal nuclei or nucleomorphs are retained in the sea slugs. While no obvious nuclei or nucleomorphs have been observed in electron microscopy studies of the animal (Graves et al., 1979; Mujer et al., 1996; Fig. 2, A and B), this does not rule out the possibility that an algal nucleus could be hiding somewhere. Recent molecular evidence employing gene probes to the multicopy ribosomal intertranscribed spacer (ITS) region of *V. litorea* nDNA also failed to detect algal nDNA in photosynthetically active *E. chlorotica* (Green et al., 2000).

The significant difference in endosymbiont longevity between chlorophyte (e.g. *Codium fragile*; Trench, 1975) and chromophyte (e.g. *V. litorea*; Mujer et al., 1996; Green et al., 2000) plastids suggests that there are some characteristics inherent in the chromophyte plastids that contribute to long-term continuous functioning. Five possibilities discussed below include: (a) there is an unusually high level of chloroplast gene autonomy in *V. litorea*; (b) chromophyte plastids and essential proteins are extremely stable; (c) the minimal protein composition needed to support the observed plastid activity is less than expected; (d) there is redirection of mitochondrial or other animal encoded proteins with the same or related function to the sea slug chloroplast; and (e) there has been lateral gene transfer from the alga to the sea slug.

Chloroplast Gene Autonomy

While the plastid genomes of chromophytes are sometimes smaller than those of chlorophytes, they typically have an increased coding capacity due to more compact coding regions and reduced inverted repeat size. A comparison of the completely sequenced chloroplast genome of the chromophyte *Odontella sinensis* and the green alga *Chlorella vulgaris* reveals a genome size/number of genes equal to 119.7 kb/174 genes and 150.6 kb/111 genes, respectively (Kowallik et al., 1995; Wakasugi et al., 1997). The increased coding capacity is reflected in an increased number of genes for the photosystem complexes and ATP synthase (*psaD*, *psaE*, *psaF*, *psaI*, *psaJ*, *psaL*, *psbV*, *psbW*, *psbX*, *petJ*, *petK*, *atpD*, and *atpG*), gene expression (22 additional ribosomal protein genes), and protein translocation/quality control (including *secY*, *secA*, *tatC* [*ycf43*], *clpC*, *ftsH* [*ycf25*], *groEL*, and *dnaK*), as well as several biosynthetic (notably *rbcS*) and hypothetical open reading frames (*ycf*). However, examples of genes considered essential for oxygenic photosynthesis and ATP synthesis that are still not expected to be plastid encoded in *V. litorea* include *petC*, *psbO*, and *atpC*. Complete se-

quencing of the *V. litorea* plastid genome is required to confirm this.

Chloroplast Stability

In the early 1970s, Trench et al. (1973) questioned whether there was something about the structure of the symbiont plastids, an unusual "robustness," that contributed to their survival outside of the algal cell. They found that isolated *Codium fragile* chloroplasts were quite stable relative to spinach chloroplasts, fixing CO₂ for at least 5 d and failing to readily rupture even when transferred to water. It was concluded that the "robustness" of the plastids might play a role in facilitating uptake of the plastids into the animal cytosol without rupture, but could not sustain their activity beyond a few days. Whether chromophytic plastids are even more stable than the green algal plastids remains to be determined. From practical experience, however, it is very easy to isolate intact plastids from *V. litorea*, and in turn quite difficult to rupture them for suborganellar characterization. We have not yet examined long-term functioning of isolated plastids.

Protein Stability and Minimal Protein Requirements

It is possible (although unprecedented) that the nuclear-encoded subunits (e.g. the light-harvesting complex proteins) are very stable and do not turn over for several months. It is also possible that our understanding of what constitutes a minimal photosystem complex or nuclear regulation of gene expression does not apply to chromophytes, and that photosystem or transcription/translation complexes lacking nuclear-encoded subunits critical in other photosynthetic organisms are actually functional.

Animal Nuclear-Encoded Proteins Redirected to the Plastids

Proteins destined for the mitochondria or involved in animal gluconeogenesis and pentose phosphate pathways may be directed to and function in the plastid. Precedence for dual targeting of proteins in a single organism has been demonstrated both in vitro (Chow et al., 1997) and in vivo (Creissen et al., 1995; Menand et al., 1998). The plastid Rieske Fe/S protein is structurally and functionally related to the nuclear-encoded mitochondrial ubiquinone-oxidoreductase complex Rieske protein. This makes it an attractive candidate for substitution by the corresponding animal nuclear gene. Another protein that could be provided by the animal genome is the regulatory γ -subunit of the ATP synthase complex encoded by *atpC*. Other prime candidates include enzymes of the photosynthetic carbon reduction (PCR) cycle. This cycle consists of gluconeogenic and pentose phosphate reactions catalyzed by enzymes found in most of the

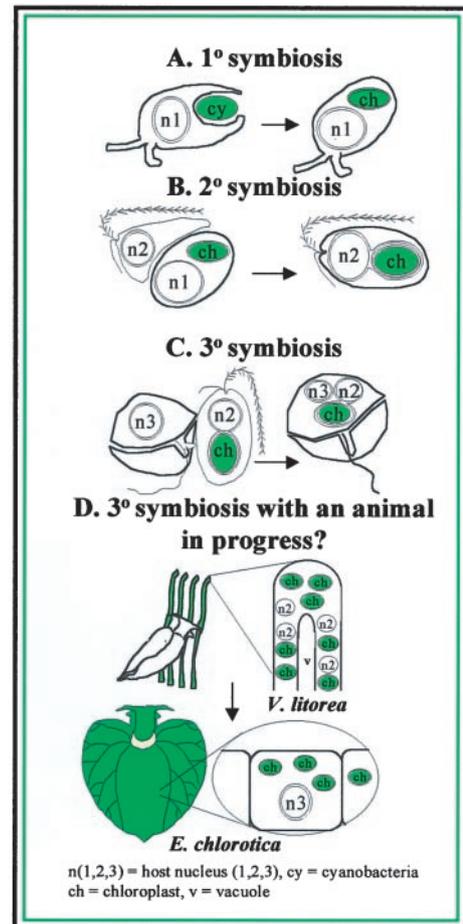


Figure 4. Symbiosis and the evolution of eukaryotic algae. Representative examples of types of endosymbiotic events responsible for multiple acquisitions of photosynthesis in eukaryotes are shown in the schematic. A, The original symbiotic event is believed to have been between a primitive flagellate protist (n1 represents the protist's nucleus) and a cyanobacteria (cy). Massive gene loss and transfer occurred between the cyanobacteria and n1, resulting in the formation of an obligate intracellular chloroplast (ch). B, Hypothetical ancestral heterotrophic stramenopile (nucleus represented by n2) incorporating a photoautotrophic eukaryotic alga. Massive gene transfer occurred between n1 and n2, resulting in the loss of n1 and divergence into the chromophytes. C, Hypothetical heterotrophic dinoflagellate (n3) incorporating a chromophyte. In this example, n2 is retained. In other symbiotic events, gene transfer between n2 and n3 occurred, resulting in n2 becoming highly reduced (nucleomorph) or lost. D, The derived chromophytic macroalga *V. litorea* undergoes symbiosis with *E. chlorotica* as a result of suctorial feeding by the juvenile sea slugs on the algal filaments. The *V. litorea* nuclei (n2) are lost, and only intact chloroplasts (ch) are retained in the cytoplasm of the cells lining the digestive diverticula (see magnification of cell layer).

biological world, with only two reactions (Rubisco and phosphoribulokinase [PRK]) unique to photosynthesis; for review, see Sharkey, 1998). Rubisco is the only plastid-encoded enzyme of the PCR cycle. In chlorophytes, only the large subunit is plastid encoded, but in all of the non-green algae studied to date, including *V. litorea* (accession no. AF207527),

both subunits of Rubisco are in the plastid genome (for review, see Kapoor and Sugiura, 1998). Since the enzymatic pathway for carbohydrate synthesis after Rubisco is basically the same in animals and plants, the sea slug can reasonably be expected to carry out these reactions up to the formation of ribulose-5-P, and possibly supply all but one (PRK, see below) of the remaining essential enzymes if appropriately targeted.

Lateral Gene Transfer

Probably the most intriguing possibility is that lateral gene transfer from *V. litorea* or a green alga to the mollusc has occurred, and that these nuclear-encoded genes are retargeted to the endosymbiont. The only enzyme in the PCR cycle unaccounted for in either the plastid genome or animal genomes is PRK. Since *E. chlorotica* has demonstrated the ability to carry out CO₂ fixation for several months, and PRK is essential for regenerating the CO₂ acceptor ribulose-1,5-bisP to keep the PCR cycle turning, the gene for PRK makes an excellent candidate for lateral gene transfer from the algal nucleus to the sea slug.

TERTIARY SYMBIOSIS OR "CAN AN ANIMAL BE AN ALGA?"

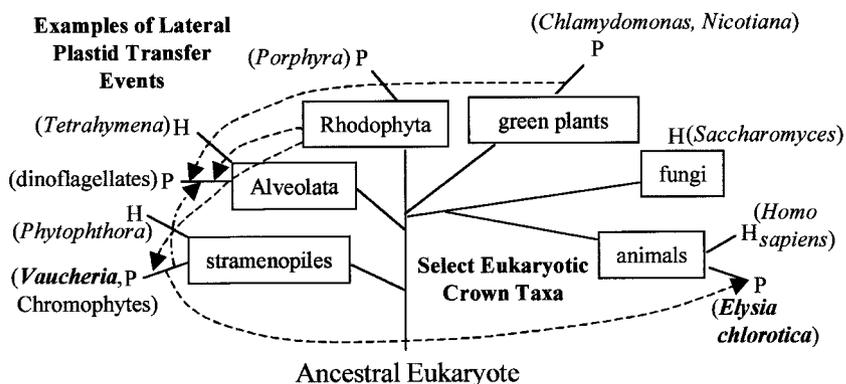
How does the association of free, functional plastids in an animal cell relate to algal biology and evolution? The cells of *E. chlorotica* are comprised of several obviously unrelated genetic systems, the animal nuclear and mitochondrial genomes and the algal chloroplast genome. This sort of structural and genetic chimerism is far from novel (Margulis, 1998). The original chloroplast was a free-living cyanobacteria that became irreversibly incorporated into the cytoplasm of a protist. Most algae are not direct descendants of this, but instead are eukaryotic chimeras as a result of a heterotrophic eukaryote incorporating a photosynthetic eukaryote (Palmer and Delwiche, 1996; Douglas, 1998; Margulis, 1998; illustrated in Fig. 4).

The evidence for eukaryotic chimerism among the algae is overwhelming, whether based on morphological or molecular data. A common diagnostic index of the eukaryotic origin of plastids is the number of membranes surrounding the plastid (Douglas, 1998). Two membranes (i.e. the typical inner and outer envelope membranes of chlorophytes) are considered diagnostic of descending from a primary symbiotic event. Three or four delineating membranes (the chloroplast endoplasmic reticulum, ctER) are considered indicative of secondary or tertiary events. Interestingly, *V. litorea* plastids are associated with multiple layers of ctER in the algal cytosol (Fig. 2, C and D), but upon engulfment by *E. chlorotica* the ctER is lost, resulting in plastids in the sea slug cytosol with only two envelope membranes (Fig. 2, A and B; Graves et al., 1979). This supports arguments that membrane reduction can occur during eukaryotic symbiosis, and that other organisms considered to be the descendants of the primary symbiotic event might be reductions of a eukaryotic symbiosis (Stiller and Hall, 1997; Van de Peer and De Wachter, 1997).

One result of this chimerism is that many algal lineages are, in terms of the non-photosynthetic cellular components such as cytosolic ribosomes, more closely related to non-photosynthetic eukaryotes than to other algal lineages. The term algae, therefore, is much more taxonomically ambiguous than the term higher plant. A simplistic way to describe these relationships is to draw a standard tree for the host based on cytosolic genes such as 18S rDNA sequences and superimpose plastid transfer events over this (Van Den Hoek et al., 1995; see Fig. 5). While chloroplast acquisition during *E. chlorotica* development has similarities to algal evolution, it can be argued that the term algae should be restricted to organisms in which the plastid is transmitted reproductively.

Whatever mechanisms are at work to maintain long-term plastid activity, the fact that *E. chlorotica* challenges the very heart of our understanding of chloroplasts is what makes this innocuous, green, solar-powered sea slug such an exciting biological system.

Figure 5. Phylogenetic tree of select eukaryotic crown taxa with diagram of lateral plastid transfer events superimposed. A simplistic diagram of representative eukaryotic taxa based on 18S rRNA sequences is indicated by the solid line. Superimposed (dashed lines) on the phylogenetic tree are lateral symbiotic events with photosynthetic eukaryotes that led to the divergence of photosynthetic (P) and heterotrophic (H) lineages within each taxon. Examples of existing members of these lineages are indicated in parentheses. Exact divergence orders and distances are not implied.



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