Transcriptomic Evidence That Longevity of Acquired Plastids in the Photosynthetic Slugs *Elysia timida* and *Plakobranchus ocellatus* Does Not Entail Lateral Transfer of Algal Nuclear Genes

Heike Wägele,*[,1] Oliver Deusch,[2] Katharina Händeler,[1] Rainer Martin,[3] Valerie Schmitt,[1] Gregor Christa,[1] Britta Pinzger,[2] Sven B. Gould,[2] Tal Dagan,[2] Annette Klussmann-Kolb,[4] and William Martin[2]

[1]Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany

[2]Institut für Botanik III, Heinrich Heine Universität Düsseldorf, Düsseldorf, Germany

[3]Z.E. Elektronenmikroskopie, Universität Ulm, Ulm, Germany

[4]Institut für Ökologie, Evolution und Diversität, Goethe-Universität Frankfurt, Frankfurt am Main, Germany

***Corresponding author:** E-mail: hwaegele@evolution.uni-bonn.de.

Associate editor: Dan Graur

Abstract

Sacoglossan sea slugs are unique in the animal kingdom in that they sequester and maintain active plastids that they acquire from the siphonaceous algae upon which they feed, making the animals photosynthetic. Although most sacoglossan species digest their freshly ingested plastids within hours, four species from the family Plakobranchidae retain their stolen plastids (kleptoplasts) in a photosynthetically active state on timescales of weeks to months. The molecular basis of plastid maintenance within the cytosol of digestive gland cells in these photosynthetic metazoans is yet unknown but is widely thought to involve gene transfer from the algal food source to the slugs based upon previous investigations of single genes. Indeed, normal plastid development requires hundreds of nuclear-encoded proteins, with protein turnover in photosystem II in particular known to be rapid under various conditions. Moreover, only algal plastids, not the algal nuclei, are sequestered by the animals during feeding. If algal nuclear genes are transferred to the animal either during feeding or in the germ line, and if they are expressed, then they should be readily detectable with deep-sequencing methods. We have sequenced expressed mRNAs from actively photosynthesizing, starved individuals of two photosynthetic sea slug species, *Plakobranchus ocellatus* Van Hasselt, 1824 and *Elysia timida* Risso, 1818. We find that nuclear-encoded, algal-derived genes specific to photosynthetic function are expressed neither in *P. ocellatus* nor in *E. timida*. Despite their dramatic plastid longevity, these photosynthetic sacoglossan slugs do not express genes acquired from algal nuclei in order to maintain plastid function.

Key words: endosymbiosis, lateral gene transfer, Sacoglossa, *Elysia*, plastid evolution, Plakobranchus, photosynthesis, EST analyses.

Introduction

Sequestration of plastids has been described in several sacoglossan sea slugs (Opisthobranchia, Gastropoda, Mollusca) (Kawaguti and Yamasu 1965; Trench et al. 1969; Waugh and Clark 1986; Marín and Ros 1989; Rumpho et al. 2001; Evertsen et al. 2007), but only four species are known to perform long-term maintenance of acquired plastids. The first of these is the well-known *Elysia chlorotica* Gould, 1870, which inhabits temperate areas of the Northwest Atlantic Ocean (Rumpho et al. 2001, 2008; Pierce et al. 2007; Schwartz et al. 2010). The second is *Plakobranchus ocellatus* (fig. 1*a*,*b*), a species with a wide distribution in shallow waters of the tropical Pacific (Wägele and Johnsen 2001; Händeler et al. 2009). The third is *Elysia timida* (fig. 1*c*), which is a typical inhabitant of shallow sublitoral zone in the Mediterranean Sea but is also reported for Caribbean and Central Eastern Atlantic localities (Wirtz and Anker 2009). Fourth, *Elysia crispata* (Mörch, 1863) (supplementary fig. 1*a*, Supplementary Material online) is restricted to the tropical Caribbean. In comparison with other sacoglossans, only the plastids of these species show a high photosynthetic rate over long periods of time (Rumpho et al. 2001; Händeler et al. 2009).

Photosynthetically functional plastids of higher plants and algae contain on the order of 2,000 proteins but only 60–200 protein-coding genes are present in the plastid across various algal groups (Timmis et al. 2004).

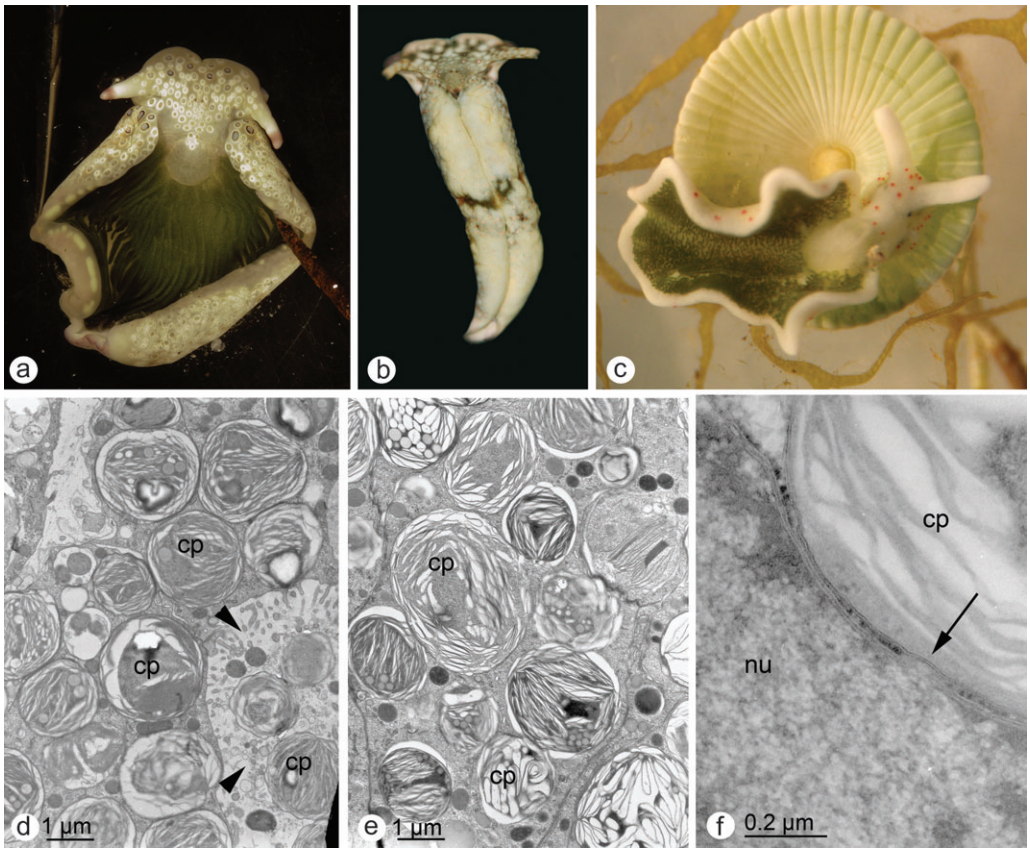

FIG. 1 Photosynthetically active sacoglossans and transmission electron micrographs (TEMs) of their plastids. (*a*) *Plakobranchus ocellatus* (Lizard Island, Australia, 4-cm long), parapodia opened to show ridges, special morphological adaptations harboring high concentrations of plastids. (*b*) *Plakobranchus ocellatus* (Guam, Mariana Islands, 1.5-cm long), parapodia closed. (*c*) *Elysia timida* (Mediterranean Sea, size ~1 cm) on the cap of *Acetabularia acetabulum*. (*d*) *Elysia timida*: TEM of plastids in digestive glandular cells directly after feeding on *A. acetabulum*. Note the plastids located in the lumen of the digestive tract (arrowheads). (*e*) *Elysia timida*: TEM of plastids in cells of digestive glandular system (2 months of starvation). (*f*) *Elysia timida*: TEM of chloroplast in close contact to nuclear pore (arrow), directly after feeding. cp, plastid; nu, nucleus.

Accordingly, plastid function is dependent upon the products of more than 1,000 nuclear-encoded genes, many, if not the majority, of which were acquired from the cyanobacterial antecedent of plastids via gene transfer from endosymbiont to host (Gould et al. 2008; Archibald 2009; Kleine et al. 2009). In the case of plastid sequestration during sacoglossan development, it has long been suspected that genes have been transferred from algae to the slugs because many components of photosystems in active algal plastids are very short-lived, with turnover times on the order of hours or less in bright light (Schuster et al. 1988; Vass et al. 1992; Warner et al. 1999). Thus, if the photosynthetic machinery is turned over quickly, but the plastids are maintained in an active state for weeks and months, algal nuclear genes can be suspected to be involved in plastid development, function, and maintenance within animals from these sacoglossan species (Rumpho et al. 2001, 2008; Pierce et al. 2007; Schwartz et al. 2010). Wishing to know more about the molecular basis of plastid retention in these photosynthetic animals, we embarked upon a deep-sequencing approach from two sacoglossans that maintain active plastids over periods of months, in search of evidence for the expression of algal nuclear genes in photosynthetic slugs.

Materials and Methods

RNA Analysis

Animals were held in ethanol/dry ice (*P. ocellatus*: 1 specimen) or liquid nitrogen (*E. timida*: 15 specimens) until being ground to a fine powder in liquid nitrogen. Powder was resuspended in 1-ml TRIzol reagent (Invitrogen), and RNA was isolated according to the manufacturer's instructions. Normalized cDNA libraries were constructed, sequenced with a 454 platform, and assembled by GATC Biotech (Konstanz, Germany). Contigs were annotated by comparison with REFSEQ. Candidate genes for transfer were identified as those having the best Blast hits in algae or plants (as opposed to animals) when compared with the REFSEQ database (Pruitt et al. 2005). A complete summary of all contig comparisons for both species is given in supplementary data 3 and 4 (Supplementary Material online). Sequences reported in this paper have been deposited with GenBank

under the accession numbers HP163845–HP241492 (*P. ocellatus*) and HP139645–HP163844 (*E. timida*).

Transmission Electron Microscopy

For transmission electron microscopy, pieces of slug parapodia were immersed in hexadecen-filled aluminum disks (bore holes 0.2-mm deep and 2-mm wide). Specimens were frozen under 2,000 bar of liquid nitrogen (Müller and Moor 1984) in a high pressure freezing (HPF) Compact 01 (Wohlwend, Switzerland). Frozen samples were immersed in 2% OsO_4 and 4% H_2O in acetone at −90 °C in a freeze substitution machine. Temperature was increased to 0 °C (within 24 h), samples were transferred into a graded series of acetone/Epon mixtures at room temperature and embedded in Epon. Sections 70-nm thick were contrasted with lead citrate.

PAM Measurements

Specimens were kept in aquariums without food at 20 °C (*E. timida*) or 22 °C (*P. ocellatus*), natural salinity, and natural light conditions (not direct sunlight) for several weeks. Measurements were taken with a Pulse Amplitude Modulated Fluorometer (Diving PAM, Walz, Germany) during the night (hence dark acclimated) with up to three measurements per individual allowing dark acclimation again after each measurement (Händeler et al. 2009).

Results and Discussion

We first examined the contents of the digestive gland cells from *P. ocellatus* and *E. timida* under the electron microscope directly after capture and after starvation for several weeks (for details of animal capture and culture, see supplementary table 1, Supplementary Material online). Chloroplasts in these species appear healthy both before and after starvation (fig. 1*d–f*). By comparison, a sacoglossan with short-term plastid retention, *Thuridilla hopei* (supplementary fig. 1*b*, Supplementary Material online), shows degrading chloroplasts in the digestive gland cells when collected (not shown) and mainly plastid remnants after 15 days of starvation (supplementary fig. 1*c*, Supplementary Material online). We found no dividing chloroplasts and no algal nuclei in any of the investigated specimens, consistent with previous findings (Greene 1970). Finding no algal nuclei is important, as it rules out the possibility that photosynthetic slugs retain transcriptionally active nuclei from their prey as shown for the ciliate *Myrionecta rubra* (Johnson et al. 2007). Sacoglossans with long-term plastid retention feed upon siphonaceous algae (Händeler and Wägele 2007) that have large cells containing many plastids and nuclei. Many siphonaceous algae, including *Codium fragile*, *C. vermilara*, *Caulerpa simpliciuscula*, and *Acetabularia acetabulum*, form a cytoplast—a membrane surrounding the plastids including cytoplasm and even mitochondria (Grant and Howard 1980; Grant and Borowitzka 1984)—when cell walls are ruptured. Here, we observed no cytoplasts surrounding sequestered plastids rather plastids were naked in the host cytosol and sometimes even appressed upon slug nuclei (fig. 1*f*).

To identify expressed genes that might have been acquired from algae, we used a deep-sequencing approach to focus on expressed genes from photosynthesizing animals. Sacoglossans ingest not only plastids but also algal nuclei, although they do not sequester the latter. Accordingly, when looking for the expression of putatively transferred genes, animals must be starved for several weeks after grazing but prior to mRNA extraction, so that the ingested algal nuclei are either excreted or completely digested. The animals that we sequenced were collected from Guam (*P. ocellatus*) or from the Mediterranean coast of France (*E. timida*) and starved in aquariums under natural light levels and day–night rhythms for several weeks, during which we measured their photosynthetic rate. Measurements showed the same slow, gradual loss of photosynthetic activity known for these species in long-term starvation experiments (Evertsen et al. 2007; Händeler et al. 2009) (fig. 2*a*,*b*). Thus, whole animals that were harvested and homogenized for mRNA extraction after 47 (*P. ocellatus*) and 28 (*E. timida*) days of starvation (supplementary table 1, Supplementary Material online) were actively photosynthetic (fig. 2*a*,*b*) but lacked detectable algal nuclei.

Pyrosequencing and assembly of normalized cDNA libraries of polyadenylated mRNA from *P. ocellatus* and *E. timida* yielded 77,648 and 24,200 contigs, respectively (table 1). Contigs were Blasted to the REFSEQ eukaryotic database to identify genes of possible algal origin. At the 10^{-10} *e*-value threshold, 5,864 of the 6,088 *Plakobranchus* contigs (96%) that found a homologue in the database found their best homologue among animals, whereas 79 (1.3%) found their best homologue among plants. Those 79 (supplementary data 1, Supplementary Material online) are potential candidates for gene acquisition from algae, but 29 of them (0.5%) correspond to 15 proteins encoded within green-algal chloroplast DNA (Martin et al. 2002; Turmel et al. 2009) and thus likely stem from the ingested plastids themselves rather than from putatively transferred genes. The remaining 50 (0.8%) correspond to a random sample of 46 nuclear-encoded genes that are widely distributed among eukaryotes, and none of which are specific to photosynthesis (supplementary data 1, Supplementary Material online). For the 2,227 *Elysia* contigs that found hits at the given threshold, 98% had their best homologue among animals and 0.7% (16 contigs) among plants. Of those 16, two (psbS and rpl2) are chloroplast-encoded genes in most algae. The remaining 14 contain no members that, by virtue of their homologue annotation, are specifically involved in photosynthesis (supplementary data 1, Supplementary Material online). A handful of prokaryotic sequences were also detected for both species, too, which is not surprising because the animals were plucked from their natural habitat.

Photosynthetic sacoglossans do not form a monophyletic clade, being dispersed among sacoglossan phylogeny instead (fig. 3). The ability to perform long-term plastid retention is thus not likely to be a uniquely derived trait rather it would appear to have arisen independently in

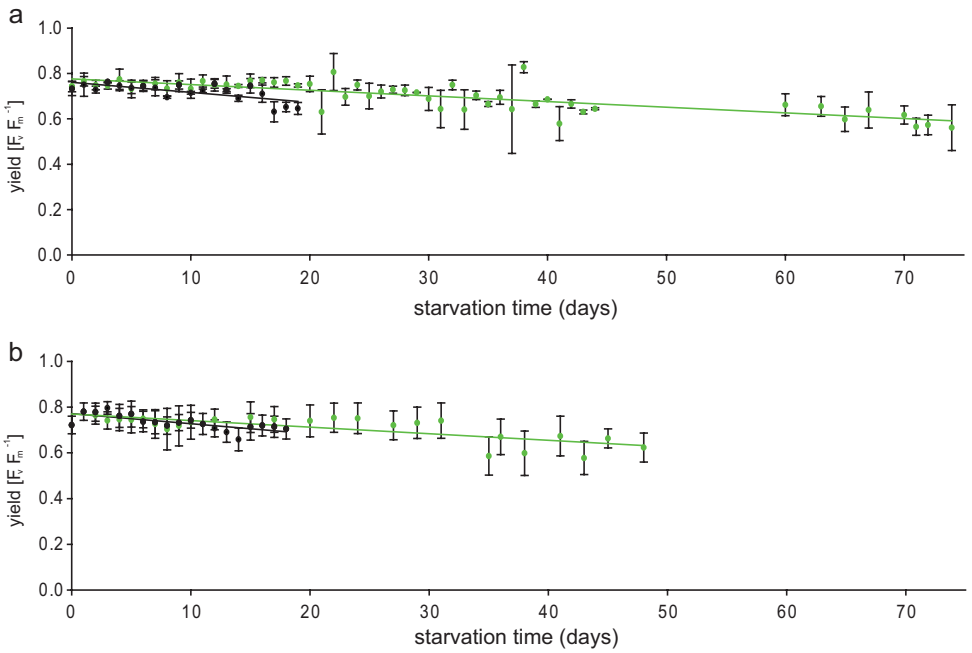

Fig. 2 Photosynthetic activity of species investigated in this study. (*a*) *Plakobranchus ocellatus* (from Australia and Guam). (*b*) *Elysia timida* (from the Mediterranean). Mean and standard deviation (usually three measurements per individual) of PAM yield values (potential quantum yield of photosystem II, F_v/F_m) are plotted against number of starvation days. Green lines (long-term measurements) show the range of photosynthetic activity observed for typical individuals collected from their natural habitats (Händeler et al. 2009). Black lines show the photosynthetic activity of the specific animals (one specimen of *P. ocellatus* and 15 specimens of *E. timida*), from which mRNA was extracted for this study.

these sacoglossan lineages. This is relevant to the nature of plastids sequestered because most photosynthetic sacoglossans prefer green-algal (chlorophyte) plastids, whereas *E. chlorotica* prefers the red algal-derived plastids of secondary symbiotic origin from the xanthophyte *Vaucheria litorea* instead (fig. 3). There are major differences in nuclear gene requirement for these two types of plastids (Gould et al. 2008; Archibald 2009). For example, chlorophyte plastids are surrounded by two membranes and require a nuclear-encoded RuBisCO small subunit (RbcS) and intrinsic antenna proteins (light harvesting complex protein [LHCP]) for function, whereas red algal-derived *Vaucheria* plastids are surrounded by four membranes—the outer two being removed by digestion in *E. chlorotica* (Rumpho et al. 2000, 2001)—encode their own RbcS, have phycobilisome-based antenna systems and additional protein targeting mechanisms. *Elysia timida* feeds on green algae (chlorophytes), with juveniles preferring *Cladophora* and adults preferring *Acetabularia* (Marin and Ros 1993). *Plakobranchus ocellatus* feeds on a wide variety of at least five species of marine ulvophyceaen chlorophytes, as revealed here by sequencing of the *tufA* gene (supplementary fig. 1*d*, Supplementary Material online), which is plastid encoded in the algal food source and by analyses of plastid-derived sequences found in the contig data (supplementary table 2, Supplementary Material online).

If genes acquired from green algae underpin photosynthesis of sequestered plastids in these sacoglossans, then nuclear genes that are highly expressed and specific to photosynthesis in the green plastid lineage should be detected. When we compare the sacoglossan contigs that identify homologues from photosynthetic eukaryotes as their best matches (table 1) to the 500 most highly expressed nuclear genes for chloroplast proteins in *Arabidopsis*, we find an almost empty set (fig. 4). Among the 500 most highly expressed nuclear genes for chloroplast proteins required for plastid function in *Arabidopsis*, we find expressed

Table 1. Summary of Taxonomic Groups with Best Matching Sequences to Sacoglossan and Control Contigs.

	Plakobranchus ocellatus		*Elysia timida*		*Acetabularia acetabulum*	
	Number	%	Number	%	Number	%
Plants and algae	79	1.3	16	0.7	715	87.0
Fungi	27	0.4	10	0.4	5	0.6
Animals	5,864	96.3	2,184	98.1	39	4.7
Prokaryotes	118	1.9	17	0.7	63	7.7
Total at 10^{-10} cutoff	6,088	100	2,227	100	822	100
Total contigs	77,648		24,200		3210	
Average contig length	663.4		554.6		n/a	
Average reads per contig	11.2		29.8		n/a	

Note.—Contigs were compared with the REFSEQ (January 2010) database using Blast, numbers indicate the number of matches at the *e*-value threshold of 10^{-10} or better. *Acetabularia* is a green alga (Ulvophyceae), the group of algae upon which the investigated sacoglossans feed and the genus upon which the investigated *E. timida* feed. The *Acetabularia* data were obtained from the GenBank EST resource and show that ulvophyceaen nuclear sequences specific to photosynthesis, had they been present in the sacoglossan data, would have readily been detected with the method used. For full details of all comparisons, see supplementary data 1–4 (Supplementary Material online). n/a, not applicable.

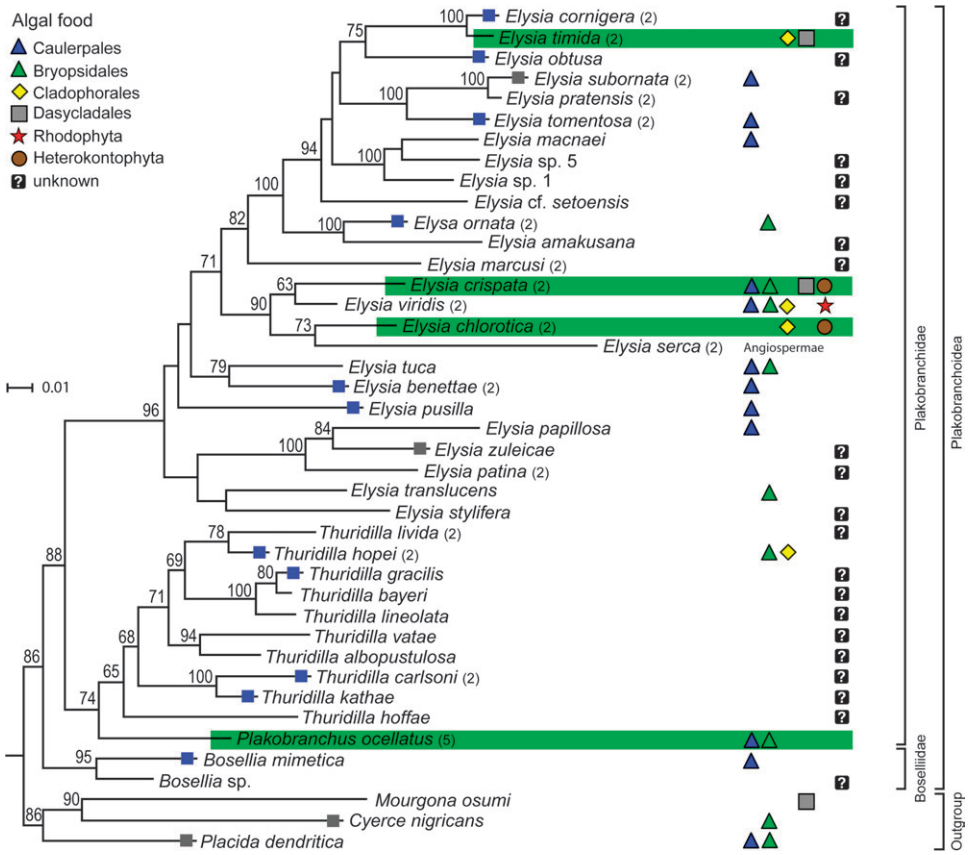

Fig. 3 Overview of phylogenetic relationships of photosynthetically active slugs within the Plakobranchidae (Sacoglossa). Maximum likelihood analysis of partial gene sequences of nuclear 28S rDNA, mitochondrial 16S rDNA, and *cox*1 (first and second codon positions) (supplementary table 3, Supplementary Material online). Numbers behind species names indicate number of individuals per species included in the analysis. Numbers at nodes indicate bootstrap values. Gray squares: species that immediately digest ingested plastids (no retention); Blue squares: species with short-term retention (up to 15 days) of sequestered plastids; Green underlain species exhibit long-term retention of functional plastids (Händeler et al. 2009). The scale bar indicates 0.1 substitutions per site. Data on food sources are combined from published data (Händeler and Wägele 2007; Händeler et al. 2009) and information collected in this study (supplementary fig. 2 and table 3, Supplementary Material online).

homologues of only two in *P. ocellatus*, a superoxide dismutase and a zinc finger protein, whereas in *E. timida* we find only one, a putative ferritin. The major nuclear-encoded proteins required by typical chlorophyte plastids, such as RbcS, LHCP, photosystem I and II components, and Calvin cycle enzymes (fig. 4) are not expressed by the slugs. We conclude that these essential plastid proteins are not provided by the slugs at all. Rather, the slugs maintain their long-lived plastids without the help of algal nuclear genes. Although our gene expression data do not thoroughly exclude the possibility that some genes might have been "transferred" from the algae to the slugs, our findings do exclude the possibility that plastid longevity in these two sacoglossans depends upon the "expression" of 1) acquired algal photosynthesis genes, germ line or otherwise, 2) sequestered algal photosynthesis genes, or 3) sequestered algal photosynthesis mRNAs because in all three cases, expressed algal nuclear genes specific to plastid photosynthetic functions should have appeared among the expressed sequence tag (EST) sequences.

This is particularly true for the most abundant nuclear-encoded proteins of plastids such as the small subunit of RuBisCO, LHCPs, or photosystem subunit proteins and the like (fig. 4, top), transcripts for which should be very abundant, should lack homologues in animals and hence would be unambiguously detectable if present. Recalling that rbcS and LHC comprise about 14% and 5%, respectively, of all transcripts in young leaves (Bhalero et al. 2003), that about one-third of the animal tissue that we harvested for sequencing is photosynthetic (fig. 1) and that a far smaller sample of only 822 *Acetabularia* ESTs at the 10^{-10} threshold (table 1) harbored expressed homologues for fully 23 of the 50 most highly expressed *Arabidopsis* nuclear-encoded chloroplast proteins (fig. 4), the absence of photosynthesis-related transcripts among the >100,000 slug contigs sampled here (averaging >10 reads per contig; >8,000 slug contigs at the 10^{-10} threshold; table 1) indicates that they do not exist in the slugs, either at levels that could relate to long-term maintenance of these photosynthetically active plastids or at all.

Our results stand in marked contrast to recent reports of gene transfer in a different sacoglossan species with long-term plastid maintenance (Pierce et al. 2007; Rumpho et al. 2008; Schwartz et al. 2010). Rumpho et al. (2008), for example, reported that the nuclear-encoded psbO gene of the xanthophyte *V. litorea* had been transferred from the algal to the *E. chlorotica* genome and is functionally

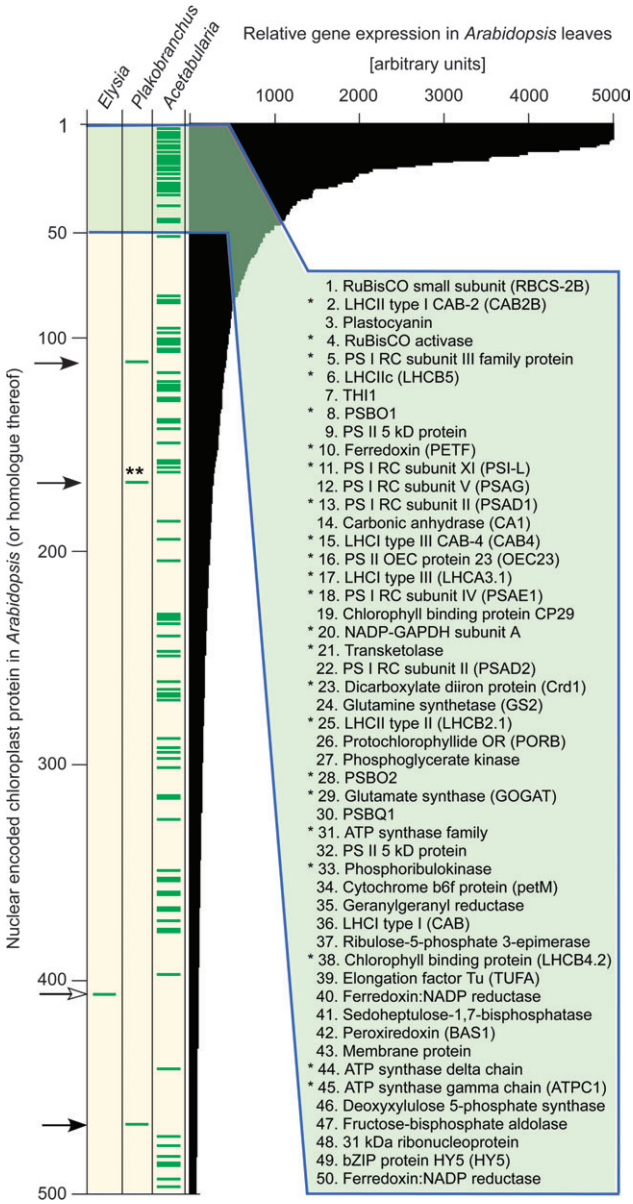

Fig. 4 Expressed genes in *Plakobranchus ocellatus* and *Elysia timida* having nuclear-encoded homologues for chloroplast proteins in *Arabidopsis*. Gene expression data (black horizontal bars) was obtained from the MPSS database (Nakano et al. 2006). Chloroplast targeting data for protein products was obtained from Tair 9 (ftp://ftp.arabidopsis.org). Genes were ranked in order of expression level (supplementary data 2, Supplementary Material online), only the 500 most highly expressed genes for nuclear-encoded chloroplast proteins in *Arabidopsis* are shown; annotations for the 50 most highly expressed genes are given in the inset. In the left-hand panels, all *E. timida* (one open arrow: ferritin) and *P. ocellatus* (three black arrows: superoxide dismutase, rpl5, and a zinc finger protein) contigs from photosynthesizing animals are indicated that found matches among the top 500 *Arabidopsis* nuclear-encoded chloroplast proteins. Double asterisk: the *Plakobranchus* contig for chloroplast ribosomal protein Rpl5 is nuclear encoded in *Arabadopsis* but plastid-encoded among algae (Martin et al. 2002). As a control, the small sample of 3,210 ESTs available for *Acetabularia acetabulum* in GenBank was compared in the same manner (*Acetabularia* column); 89 *Acetabularia* contigs were detected among the top 500, 23 *Acetabularia* contigs were detected among the top 50 nuclear-encoded chloroplast proteins in *Arabidopsis* (asterisk). Details are given in supplementary data 2–4 (Supplementary Material online). Relative expression levels for the top seven genes exceed 5,000 and are truncated here for convenience. PS, photosystem; RC, reaction center; THl 1, thiazole biosynthetic enzyme; PSBO, oxygen evolving complex protein; OEC, oxygen evolving complex.

expressed there. The targeting of this gene product to the plastid, if the gene has indeed been transferred into and expressed from the slug nuclear genome, entails considerable difficulties, however, because of the different number of membranes surrounding *V. litorea* plastids in the alga and in the slug: Whereas *V. litorea* plastids are surrounded by four membranes in the alga, the outermost two of those membranes are digested during kleptoplasty in the slug (Rumpho et al. 2001). Hence, if psbO has been transferred in *E. chlorotica*, how does the protein get inside the kleptoplast? The topogenic signal reported by Rumpho et al. (2008) resembles the typical bipartite leader sequence conserved among organisms harboring plastids of red algal origin (Gould et al. 2006). The signal and transit peptide are responsible for the targeting across a series of four translocons, best characterized in some algae having plastids with four membranes (Sommer et al. 2007; Bullmann and Maier 2010). In all such cases studied so far, the signal peptide's sole purpose is targeting across the outermost, endoplasmatic reticulum-derived plastid membrane through a SEC61-based machinery. *Elysia chlorotica* digests the two outermost membranes during the isolation of the plastids. This alters the topogenic signal requirements, as the signal peptide is no longer needed, in fact counterproductive. Signal peptides are highly conserved across eukaryotes and are recognized across many tested species (Nielsen et al. 1997). Hence, if the psbO gene was transferred as described (Rumpho et al. 2008) and then transcribed in the slug nucleus and translated in the cytosol, as it is in the intact alga, the psbO products could be directed to the *Elysia* ER. As *Vaucheria* plastids are not surrounded by ER in *E. chlorotica* (Rumpho et al. 2000, 2001), the question of how a nuclear-encoded psbO (or other) gene product reaches the plastids in *E. chlorotica*, if indeed any genes have been transferred at all in that species, is severe.

It has long been known that genes are often transferred from symbiont to host chromosomes in the wake of organelle origins during endosymbiosis (Martin 2003; Elias and Archibald 2009). More recently, it has become popular to suggest that gene transfer from food to feeder is a normal genetic consequence of having a meal (Keeling and Palmer 2008), regardless of whether organelles become established or not. The case of sacoglossans puts that theory of "you are what you eat" to it the test because the physical interactions between food plastids and their feeding host could hardly be more intimate. Many expressed genes were transferred from the cyanobacterial endosymbiont to its host during the origin of plastids (Martin et al. 2002; Archibald 2009). But there is currently no evidence in the present data to suggest that expressed genes are transferred during ontogenetic plastid acquisition from food in these

sacoglossan slugs. Thus, although the endosymbiotic origins of organelles is important in evolution and does entail gene transfer (Timmis et al. 2004), sacoglossans are not, in genetic terms, what they eat.

Even greater kleptoplast longevity has been shown for the foraminifer, *Nonionella stella*, which incorporates the chloroplasts from a diatom (Grzymski et al. 2002). Here, the proteins RuBisCO and D1 as well as the pigments fucoxanthin and chlorophyll *a* are stable for up to 1 year, revealing an extremely low turnover of that plastid machinery. However, *Nonionella* lives in much deeper water (down to 600 m) than sacoglossans, which live down to depths of only about 10–20 m. The extremely low irradiance that *Nonionella* plastids encounter reduces photodamage compared with the sacoglossans and might affect longevity. Moderate longevity of proteins might also explain the immunological detection of, for example, the chlorophyll-binding protein FCP in *E. crispata* (Pierce et al. 2003), as a simple alternative to the eukaryote-to-eukaryote lateral gene transfer hypothesis.

For two of the four known slug species that are able to maintain kleptoplasts long term, our findings resolve a long-standing question: Survival of sacoglossan plastids is attributable to proteins possessed by the plastids themselves and may also involve the intracellular milieu provided by those slug species that refrain from digesting their plastids, but it does not involve the expression of slug nuclear genes that were acquired from algae encoding photosynthetic functions. This sets a new context for the study of the adaptations—both algal and animal—that support the longevity of these animals' photosynthetic loot.

Supplementary Material

Supplementary data, figures 1–2, and tables 1–3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We thank Britta Rohde and Peter Schupp (Guam) for their support in cultivating *Plakobranchus ocellatus* specimens on Guam. Frank Richter (Chemnitz) kindly ordered and sent us *Plakobranchus* specimens from the Philippines. Paul Walther and Kathi Höhn (University of Ulm, Germany) advised and helped with high-pressure cryo fixation and Karl-Heinz Tomaschko in processing transmission electron micrographs. This work was funded through grants from the German Science Foundation to H.W. (Wa618/12-1) and an advanced grant from the European Research Council to W.M. Author contributions: H.W. conceived the project, collected animals, performed PAM measurements, and wrote the paper; O.D. analyzed the EST data, K.H. performed PAM measurements and phylogenetic analysis, R.M. performed the transmission electron micrograph (TEM) analyses, V.S. collected animals, performed PAM measurements, and helped in TEM analyses, G.C. and T.D. performed phylogenetic analyses for the food of *Plakobranchus*, B.P. extracted mRNA and prepared samples for further processing, S.B.G. helped writing the paper, A.K.-K. conceived the project and helped writing the paper, W.M. conceived the project, analyzed the EST data, and wrote the paper. Figures were prepared by H.W., K.H., G.C., and W.M.

References

Archibald JM. 2009. The puzzle of plastid evolution. *Curr Biol*. 19:R81–R88.

Bhalero R, Keskitalo J, Sterky F, et al. (11 co-authors). 2003. Gene expression in autumn leaves. *Plant Physiol*. 131:430–442.

Bullmann L, Maier UG. 2010. Filling the gap, evolutionary conserved Omp85 in plastids of chromalveolates. *J Biol Chem*. 285:6848–6856.

Elias M, Archibald JM. 2009. Sizing up the genomic footprint of endosymbiosis. *BioEssays* 31:1273–1279.

Evertsen J, Burghardt I, Johnsen G, Wägele H. 2007. Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. *Mar Biol*. 151:2159–2166.

Gould SB, Sommer MS, Hadfi K, Zauner S, Kroth PG, Maier UG. 2006. Protein targeting into the complex plastid of cryptophytes. *J Mol Evol*. 62:674–681.

Gould SB, Waller RR, McFadden GI. 2008. Plastid evolution. *Annu Rev Plant Biol*. 59:491–517.

Grant BR, Borowitzka MA. 1984. The chloroplasts of giant-celled and coenocytic algae: biochemistry and structure. *Bot Rev*. 50:267–307.

Grant BR, Howard RJ. 1980. Kinetics of C-14 distribution during photosynthesis by chloroplast preparations isolated from the siphonous alga *Caulerpa simpliciuscula*. *Plant Physiol*. 66:29–33.

Greene R. 1970. Symbiosis in sacoglossan opisthobranchs: symbiosis with algal chloroplasts. *Malacol* 19:357–368.

Grzymski J, Schofield OM, Falkowski PG, Berhard JM. 2002. The function of plastids in the deep-sea benthic foraminifer, *Nonionella stella*. *Limnol Oceanogr*. 47:1569–1580.

Händeler K, Grzymbowski Y, Krug PJ, Wägele H. 2009. Functional chloroplasts in metazoan cells—a unique evolutionary strategy in animal life. *Front Zool*. 6:28.

Händeler K, Wägele H. 2007. Preliminary study on molecular phylogeny of Sacoglossa and a compilation of their food organisms. *Bonn zool Beitr*. 55:231–254.

Johnson MD, Oldach D, Delwiche CF, Stoecker DK. 2007. Retention of transcriptionally active cryptophyte nuclei by the ciliate *Myrionecta rubra*. *Nature* 445:426–428.

Kawaguti S, Yamasu T. 1965. Electron microscopy on the symbiosis between an elysioid gastropod and chloroplasts of a green alga. *Biol J Okayama Univ*. 11:57–65.

Keeling PJ, Palmer JD. 2008. Horizontal gene transfer in eukaryotic evolution. *Nat Rev Genet*. 9:605–618.

Kleine T, Maier UG, Leister D. 2009. DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis. *Annu Rev Plant Biol*. 60:115–138.

Marín A, Ros J. 1989. The chloroplast-animal association in four Iberian sacoglossan opisthobranchs: *Elysia timida*, *Elysia translucens*, *Thuridilla hopei* and *Bosellia mimetica*. *Sci Mar*. 53:429–440.

Marin A, Ros J. 1993. Ultrastructural and ecological aspects of the development of chloroplast retention in the Sacoglossan gastropod *Elysia timida*. *J Mollusc Stud*. 59:95–104.

Martin W. 2003. Gene transfers from organelles to the nucleus: frequent and in big chunks. *Proc Natl Acad Sci U S A*. 100:8612–8614.

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M, 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 U S A*. 99:12246–12251.

Müller M, Moor H. 1984. Cryofixation of suspensions and tissues by propane jet freezing and high pressure freezing. In: Bailey GW, editor. Proceedings of the 42nd Annual Meeting Electron Microscopic Society Am, San Francisco, CA.

Nakano M, Nobuta K, Vermaraju K, Singh Tey S, Skogen JW, Meyers BC. 2006. Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Res*. 34:D731–D735.

Nielsen H, Engelbrecht J, Brunak S, von Heijne G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng*. 10:1–6.

Pierce SK, Curtis NE, Hanten JJ, Boerner SL, Schwartz JA. 2007. Transfer, integration and expression of functional nuclear genes between multicellular species. *Symbiosis* 43:57–64.

Pierce SK, Massey SE, Hanten JJ, Curtis NE. 2003. Horizontal transfer of functional nuclear genes between multicellular organisms. *Biol Bull*. 204:237–240.

Pruitt KD, Tatusova T, Maglott DR. 2005. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res*. 33:D501–D504.

Rumpho ME, Summer EJ, Green BJ, Fox TC, Manhart JR. 2001. Mollusc/algal chloroplast symbiosis: how can isolated chloroplasts continue to function for month in the cytosol of a sea slug in the absence of an algal nucleus? *Zoology*. 104:303–312.

Rumpho ME, Summer EJ, Manhart JR. 2000. Solar-powered sea slugs. Mollusc/algal chloroplast symbiosis. *Plant Physiol*. 123: 29–38.

Rumpho ME, Worful J, Lee J, Kannan K, Tyler MS, Bhattacharya D, Moustafa A, Manhart JR. 2008. Horizontal gene transfer of the algal nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica*. *Proc Natl Acad Sci U S A*. 105:17867–17871.

Schuster G, Timberg R, Ohad I. 1988. Turnover of thylakoid photosystem II proteins during photoinhibition of *Chlamydomonas reinhardtii*. *Eur J Biochem*. 1777:403–410.

Schwartz JA, Curtis NE, Pierce SK. 2010. Using algal transcriptome sequences to identify transferred genes in the sea slug, *Elysia chlorotica*. *Evol Biol*. 37:29–37.

Sommer MS, Gould SB, Lehmann P, Gruber A, Przyborski JM, Maier U-G. 2007. Der1-mediated preprotein import into the periplastid compartment of chromalveolates? *Mol Biol Evol*. 24:918–928.

Timmis JN, Ayliffe MA, Huang CY, Martin W. 2004. Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nat Rev Genet*. 5:123–135.

Trench RK, Greene RW, Bystrom BG. 1969. Chloroplasts as functional organelles in animal tissue. *J Cell Biol*. 42:404–417.

Turmel M, Otis C, Lemieux C. 2009. The chloroplast genomes of the green algae *Pedinomonas minor*, *Parachlorella kessleri*, and *Oocystis solitaria* reveal a shared ancestry between the Pedinomonadales and Chlorellales. *Mol Biol Evol*. 26: 2317–2331.

Vass I, Styring S, Hundal T, Koivuniemi A, Aro E-M, Andersson B. 1992. Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced QA species promote chlorophyll triplet formation. *Proc Natl Acad Sci U S A*. 89: 1408–1412.

Wägele H, Johnsen G. 2001. Observations on the histology and photosynthetic performance of "solar-powered" opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae. *Org Divers Evol*. 1:193–210.

Warner ME, Fitt WK, Schmidt GW. 1999. Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. *Proc Natl Acad Sci U S A*. 96:8007–8012.

Waugh GR, Clark KB. 1986. Seasonal and geographic variation in chlorophyll level of *Elysia tuca* (Ascoglossa: Opisthobranchia). *Mar Biol*. 92:483–487.

Wirtz P, Anker A. 2009. Range extension for *Elysia timida*(Opisthobranchia: Sacoglossa) to São Tomé Island (eastern central Atlantic), with a film showing the curious locomotion of the species. *Mar Biodivers Rec*. 2:e144.