MBE Advance Access published September 9, 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

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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. While 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 time scales 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.

Introduction

Sequestration of plastids has been described in several sacoglossan sea slugs (Opisthobranchia, Gastropoda, Mollusca) (Waugh and Clark 1986; Kawaguti and Yamasu 1965; Trench, Greene and Bystrom 1969; 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 NW Atlantic Ocean (Rumpho et al. 2001, 2008; Pierce et al. 2007; Schwartz, Curtis and Pierce 2010). The second is *Plakobranchus ocellatus* (Fig. 1a-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. 1c), 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 Figure 1a) is restricted to the tropical Caribbean. In comparison to 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 2000 proteins, but only 60-200 protein coding genes are present in the plastid across various algal groups (Timmis et al. 2004). Accordingly, plastid function is dependent upon the products of more than 1000 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, Waller and McFadden 2008; Kleine, Maier and Leister 2009; Archibald 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, Timberg and Ohad 1988; Vass et al. 1992; Warner, Fitt and Schmidt 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, Curtis and Pierce 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.

Results and Discussion

We first examined the contents of the digestive gland cells from *Plakobranchus ocellatus* and *Elysia 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). Chloroplasts in these species appear healthy both before and after starvation (Fig. 1d-f). By comparison, a sacoglossan with short-term plastid retention, *Thuridilla hopei* (Supplementary Figure 1b), shows degrading chloroplasts in the digestive gland cells when collected (not shown) and mainly plastid remnants after 15 days of starvation (Supplementary Figure 1c). We found no dividing chloroplasts and no algal nuclei in any of the investigated specimens, consistent with previous findings (Green 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. 1f).

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 (*Plakobranchus ocellatus*) or from the Mediterranean coast of France (*Elysia 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. 2a-b). Thus, whole animals that were harvested and homogenized for mRNA extraction after 47 (*Plakobranchus ocellatus*) and 28 (*Elysia timida*) days of starvation (Supplementary Table 1) were actively photosynthetic (Fig. 2a-b), but lacked detectable algal nuclei.

Pyrosequencing and assembly of normalized cDNA libraries of polyadenylated mRNA from *Plakobranchus ocellatus* and *Elysia 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, while 79 (1.3%) found their best homologue among plants. Those 79 (Supplementary Data 1) 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, Otis and Lemieux 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). 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). A handful of prokaryotic sequences were also detected

for both species, too, which is not surprising, since 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 these sacoglossan lineages. This is relevant to the nature of plastids sequestered, since most photosynthetic sacoglossans prefer green-algal (chlorophyte) plastids, while *Elysia 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, Waller and McFadden 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. *E. 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 Figure 1d), which is plastid-encoded in the algal food source, and by analyses of plastid-derived sequences found in the contig data (Supplementary Table 2).

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 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 homologues of only two in *Plakobranchus ocellatus*, a superoxide dismutase and a zinc finger protein, while in *Elysia 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. While 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 i) acquired algal photosynthesis genes, germline or otherwise, ii) sequestered algal photosynthesis genes, or iii) sequestered algal photosynthesis mRNAs, because in all three cases, expressed algal nuclear genes specific to plastid photosynthetic functions should have appeared among the EST sequences.

This is particularly true for the most abundant nuclear encoded proteins of plastids such as the small subunit of RuBisCO, light harvesting complex (LHC) proteins, 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 out 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; >8000 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 *Vaucheria litorea* had been transferred from the algal to the *E. chlorotica* genome and is functionally 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: While *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 harbouring 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 et al. 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. *E. 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, while 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 one 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 to 20 m. The extremely low irradiance that *Nonionella* plastids encounter reduces photodamage compared to the sacoglossans, and might affect longevity. Moderate longevity of proteins might also explain the immunological detection of e.g, the chlorophyll binding protein FCP in *Elysia 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 longstanding 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.

Materials and Methods

RNA analysis

Animals were held in ethanol/dry ice (*Plakobranchus ocellatus* 1 specimen) or liquid nitrogen (*Elysia timida:* 15 specimens) until being ground to a fine powder in liquid nitrogen. Powder was re-suspended 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 to REFSEQ. Candidate genes for transfer were identified as those having the best BLAST hits in algae or plants (as opposed to animals) when compared to the REFSEQ database (Pruitt, Tatusova and Maglott 2005). A complete summary of all contig comparisons for both species is given in Supplementary Data 3 and 4. Sequences reported in this paper have been deposited with GenBank under the accession numbers HP163845 – HP241492 (*Plakobranchus ocellatus*) and HP139645 – HP163844 (*Elysia timida*).

Transmission electron microscopy

For transmission electron microscopy, pieces of slug parapodia were immersed in hexadecen-filled aluminium disks (bore holes 0.2 mm deep and 2 mm wide). Specimens were frozen under 2000 bar of liquid nitrogen (Müller and Moor 1984) in a HPF Compact 01 high pressure freezing machine (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 (*Elysia timida*) or 22 °C (*Plakobranchus 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).

Acknowledgements

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. analysed the EST data, K. H. performed PAM measurements and phylogenetic analysis, R. M. performed the 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. G. helped writing the paper, A. K.-K. conceived the project and helped writing the paper, W. M. conceived the project, analysed the EST data and wrote the paper. Figures were prepared by H. W., K. H., G. C. and W. M.

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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	5864	**96.3**	2184	**98.1**	39	**4.7**
Prokaryotes	118	1.9	17	0.7	63	7.7
Total at 10^{-10} cutoff	6088	100	2227	100	822	100
Total contigs	77648		24200		3210	
Avg. contig length	663.4		554.6		n.a.	
Avg. reads per contig	11.2		29.8		n.a.	

Contigs were compared to the REFSEQ (Jan 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 *Elysia 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.

Figure legends

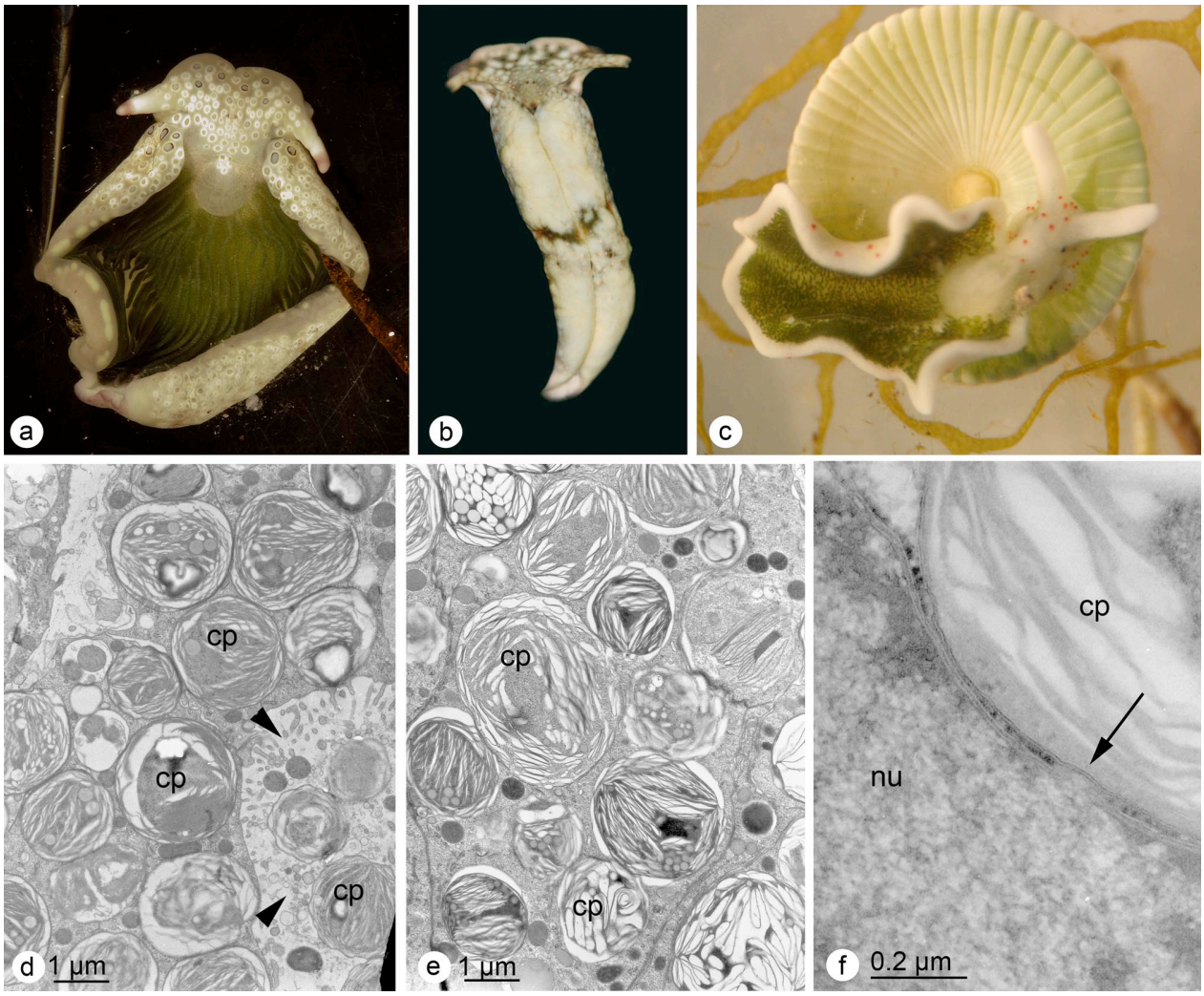

Figure 1 | Photosynthetically active sacoglossans and transmission electron micrographs (TEM) of their plastids. **a**, *Plakobranchus ocellatus* (Lizard Island, Australia, 4 cm long), parapodia opened to show ridges, special morphological adaptations harbouring high concentrations of plastids. **b**, *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 *Acetabularia 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 (two months of starvation). **f**, *Elysia timida*: TEM of chloroplast in close contact to nuclear pore (arrow), directly after feeding. cp: plastid.

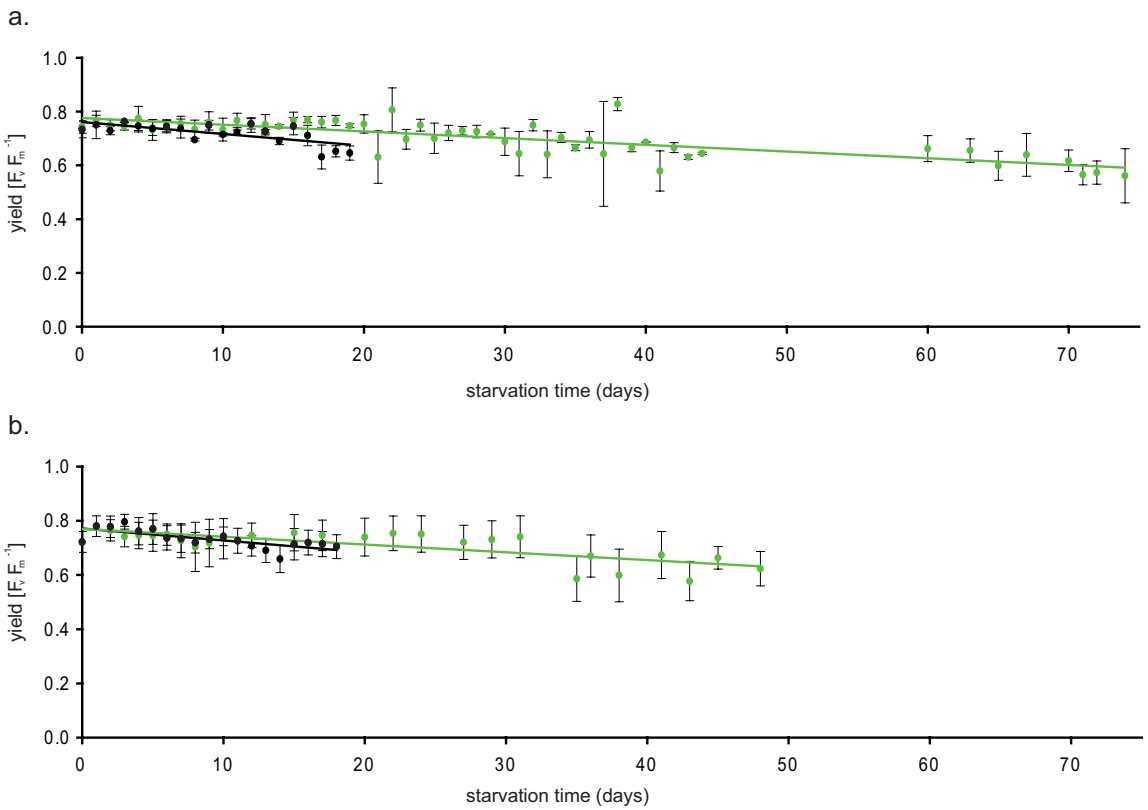

Figure 2 | **Photosynthetic activity of species investigated in this study. a,** *Plakobranchus ocellatus* (from Australia and Guam). **b**, *Elysia timida* (from the Mediterranean). Mean and standard deviation (usually three measurements per individual) of PAM yield values (potential quantum yield of 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.

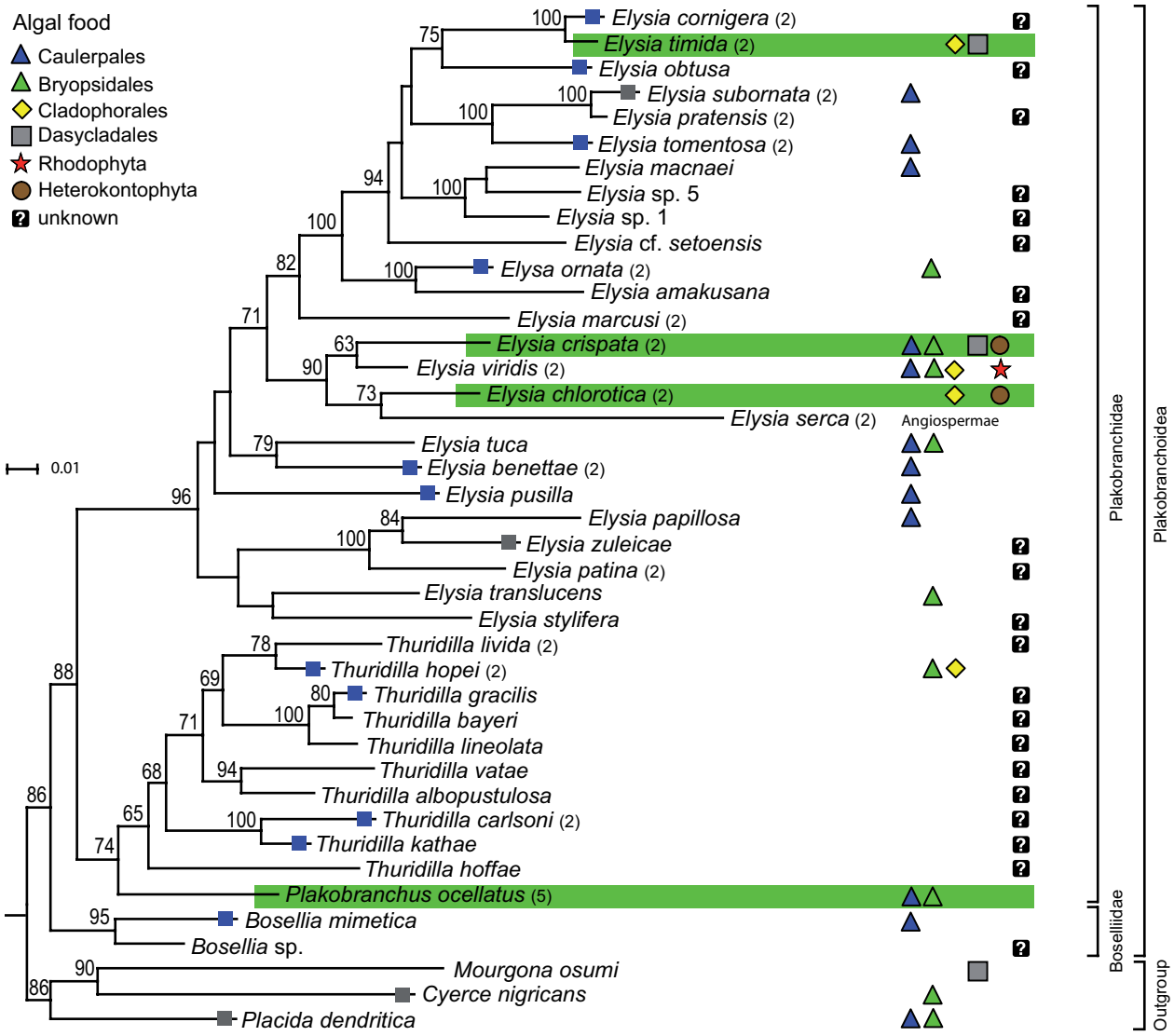

Figure 3 | Overview of phylogenetic relationships of photosynthetically active slugs within the Plakobranchoidea (Sacoglossa). Maximum likelihood analysis of partial gene sequences of nuclear 28S rDNA, mitochondrial 16S rDNA and *cox*1 (first and second codon positions) (Supplementary Table 3). Numbers behind species names indicate number of individuals per species included in the analysis. Numbers at nodes indicate bootstrap values. Grey 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 Figure 2, Supplementary Table 3).

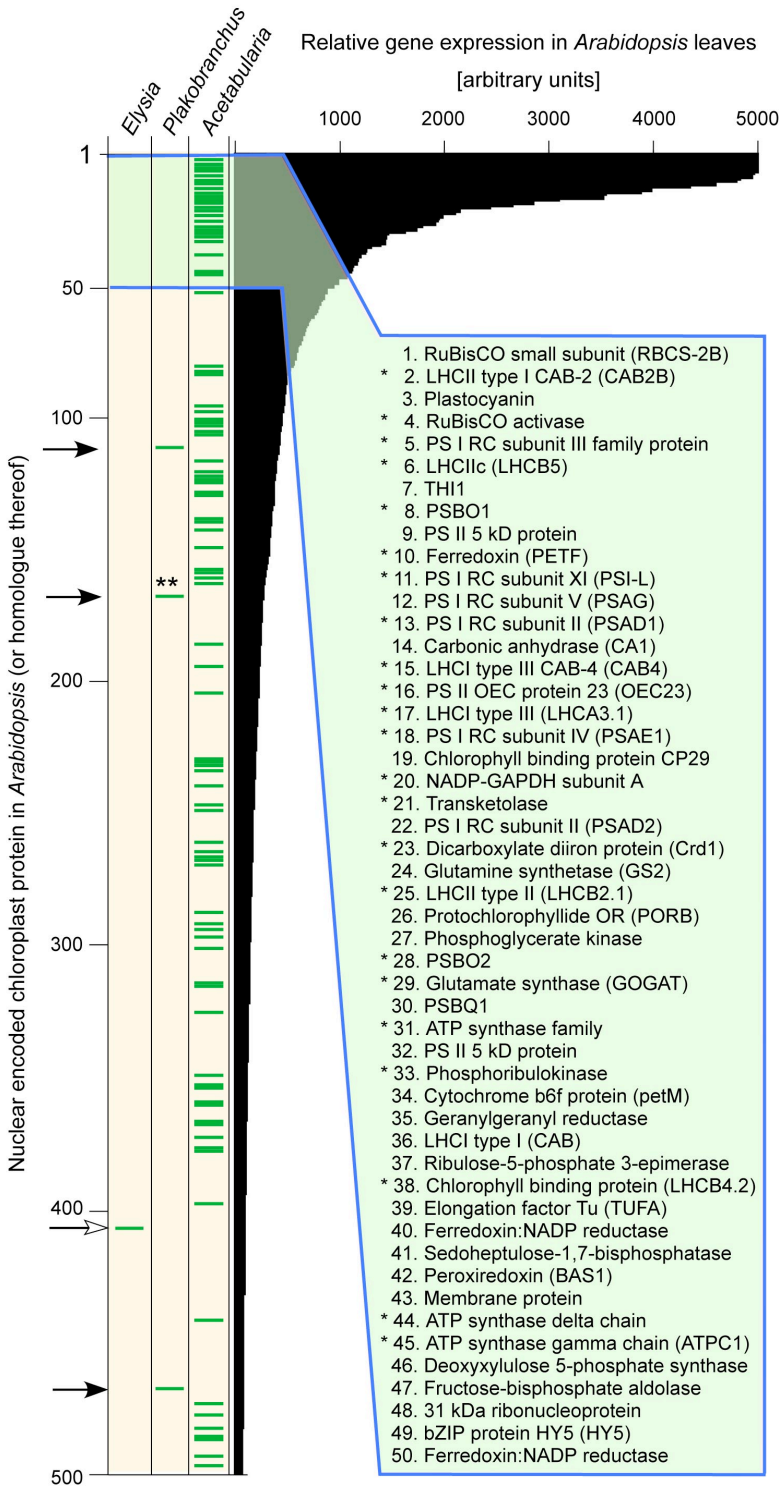

Fig. 4

Figure 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), 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 lefthand panels, all *Elysia timida* (one open arrow: ferritin) and *Plakobranchus 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 3210 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. Relative expression levels for the top seven genes exceed 5000 and are truncated here for convenience. LHC, light harvesting complex; PS, photosystem, RC, reaction center, THI1, thiazole biosynthetic enzyme; PSBO, oxygen evolving complex protein; OEC, oxygen evolving complex.